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Effect of a single coding polymorphism in the *PRNP* gene on PrP^{Sc} levels in prion-infected sheep lymphoid tissues.

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MSc by Research Neurobiology

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Abstract

Sheep orally infected with bovine spongiform encephalopathy (BSE) have been employed as a model for analysing the risk of transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Previously, our group has shown that the probability of BSE transmission (via blood transfusion) correlates with the *PRNP* codon 141 genotypes in donor sheep. Since the lymphoreticular system has been established as an important site for disease-related PrP^{Sc} replication, we speculated that the titre of infectivity in the blood depends on the codon 141 genotype and/or levels of PrP^{Sc} in the lymphoid tissues. Since PrP^{Sc} is often employed as one of the markers for TSEs infectivity, the purpose of this project was to measure and compare PrP^{Sc} levels in lymphoid tissues from sheep with different *PRNP* codon 141 genotypes. ELISA (TeSeE™ sheep/goat kit) was adapted to quantify PrP^{Sc} in the brain and lymphoid tissues by using truncated recombinant ovine $A_{136}R_{154}Q_{171}$ PrP as a standard to estimate relative PrP^{Sc} level. In the spleen and prescapular lymph nodes, PrP^{Sc} concentrations were similar for LL_{141} and FF_{141} sheep, but in LF_{141} sheep were consistently observed below the TeSeE sheep/goat kit cut-off value (< 2.4ng/mg), suggesting that there is substantially reduced PrP^{Sc} deposition in lymphoid tissues. This finding showed codon 141 genotype has no distinct impact on PrP^{Sc} deposition in the brain regardless of genotype. The finding indicated that the amount of PrP^{Sc} deposited in lymphoid tissues is related to codon 141 genotypes. The effect of genotype on PrP^{Sc} deposition will be correlated to the bioassay infectivity through seeding rate determined from an *in vitro* conversion method (real-time quaking-induced conversion assay, RT-QuIC).

Lay summary

Sheep infected with mad cow disease or bovine spongiform encephalopathy (BSE) via ingestion has been used as a model for analysing the risk of transmitting human variant Creutzfeldt-Jakob disease via blood transfusion. Previously we found that BSE transmission was affected by genetic variation of the amino acid at position 141 for PrP protein gene. Our hypothesis was that the concentration of disease infectivity in the blood depends on the genetic sequence of the amino acid at position 141 (PrP protein gene) and/or the amount of infectivity in the lymphoid tissues, as they have been proven to be important sites for disease-related PrP^{Sc} amplification. As PrP^{Sc} is the major markers for infectivity, the aim of the project is to measure and compare PrP^{Sc} concentration in lymphoid tissues from sheep that differ in PrP protein gene's amino acid at position 141. A commercially available diagnostic kit (TeSeE™ sheep/goat kit) was adapted to quantify PrP^{Sc} in the brain and lymphoid tissues, using artificial sheep PrP protein as the standard for estimation of relative PrP^{Sc} concentrations. The results indicated that the amount of PrP^{Sc} in lymphoid tissues is related to the amino acid type at position 141 of the PrP protein. In the spleen and prescapular lymph node samples, PrP^{Sc} concentrations were similar when the amino acids at position 141 (PrP protein) were identical, but were consistently below the detectable range in the samples with non-identical amino acids at position 141. In future experiments, we will explore the relationship between PrP^{Sc} concentration and infectivity with the artificial amplification method called RT-QuIC.

Contents

Abbreviations

263K	Hamster-adapted scrapie strain
4PL	Four-parameter logistic
79A	Mouse-adapted scrapie strain
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CD	Cluster of differentiation with derivatives of CD21 (known as Epstein-Barr virus receptor) and CD62 (known as selectins) mentioned in the introduction.
CJD	Creutzfeldt-Jakob disease with derivatives of variant CJD (vCJD), sporadic CJD (sCJD), familial CJD (fCJD) or iatrogenic CJD (iCJD)
CNS	Central nervous system
CSF	Cerebrospinal fluid
C_t	Cycle threshold
CV	Coefficient of variation
CWD	Chronic wasting disease
DC	Dendritic cells with derivative of follicular DC (FDC)
DDA	Direct detection assay
DEFRA	Department for environment, food & rural affairs
DMNV	Dorsal motor nucleus of the vagus nerve
dpi	days' post inoculation
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay with derivative of enzyme-linked immunosorbent assay (ELISA)
ENS	Enteric nervous system
EUE	Exotic ungulate encephalopathy
FFI	Fatal familial insomnia
FSE	Feline spongiform encephalopathy
GALT	Gut-associated lymphoid tissue with derivatives of distal ileal Peyer's patches (DiPP only in sheep), mesenteric lymph nodes (MLN).
GC	Germinal centre
GPI	Glycosylphosphatidylinsitol glycolipid
GSS	Gerstmann-Sträussler-Scheinker syndrome
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HSrPrP	Hamster-sheep chimeric recombinant PrP protein
IHC	Immunohistochemistry (assay)
IP	Immunoprecipitation (assay)
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LD_{50}	Median lethal dose
LRS	Lymphoreticular system
LTβR-Ig	lymphotoxin β receptor-immunoglobulin
MBM	Meat and bone meal
M cells	Microfold cells
MgCl	Magnesium chloride
MES	2-(N-morpholino) ethanesulfonic acid
MicroBCA	Microplate bicinchoninic acid (assay)
MSC II	Microbiological safety cabinet class 2

NaCl	Sodium chloride
NaOH	Sodium hydroxide
$NaPO_4$	Sodium phosphate
NaPTA	Sodium phosphotungstic acid
na	not applicable
nd	not done
NHSBT	NHS blood and transplant
Ni-NTA	Nickle nitrilotriacetic acid
OD	Optical density
oor	Out of range
PAGE	Polyacrylamide gel electrophoresis (assay)
PBS	Phosphate-buffered saline
PK	Proteinase K
PLT	Platelets
PMCA	Protein misfolding cyclic amplification with derivative of serial PMCA (sPMCA).
PMSF	Phenylmethanesulfonyl fluoride
PNS	Peripheral nervous system
PRNP	Prion protein
PrP^C	Cellular PrP protein (not associated with disease)
PrP^{Sc}	Scrapie PrP protein (disease associated form) also known as PrP^D
PSLN	Prescapular lymph nodes
PVDF	Polyvinylidene difluoride
RFU	Relative fluorescence unit
rPrP	recombinant PrP protein also refers to truncated ovine $A_{136}R_{154}Q_{171}$ rPrP in this study
rpm	Revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction (assay)
QuIC	quaking-induced conversion (assay) with derivatives of real-time QuIC (RT-QuIC) and enhanced QuIC (eQuIC).
S1PR1	Sphingosine 1-phosphate receptor
SD	Standard deviation
SD_{50}	Median seeding dose
SDS	Sodium dodecyl sulphate
TBMs	Tingible body macrophages
ThT	Thioflavin T
TIFF	Tagged image file format
TME	Transmissible mink encephalopathy
TP	Total protein
TSEs	Transmissible spongiform encephalopathies
UV	Ultraviolet-visible
WB	Western blot (assay)
WBC	White blood cells also termed leucocytes with derivatives of peripheral blood mononuclear cells (PBMC), B cells and T cells

Introduction

Transmissible spongiform encephalopathies (TSEs) and the role of PrP^{Sc}

Transmissible spongiform encephalopathies, or prion diseases, are fatal neurodegenerative disorders that affect various mammalian species including classical scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer, sporadic and variant Creutzfeldt-Jacob disease (sCJD, vCJD) in humans. TSE diseases are characterised by long incubation times (may be up to decades in humans), and the development of vacuolar lesions in the central nervous system (CNS), neuronal loss, and accumulation of protein aggregates occurring during disease progression (*1*). Affected animals and humans generally display behavioural changes such as cerebral ataxia, cognitive decline in human and motor dysfunction. The causative agent of TSE disease is hypothesised to be a misfolded form of PrP protein (*1*).

Normal cellular PrP protein (PrP^{C}) is a detergent soluble glycosylphosphatidylinositol (GPI) protein with predominantly alpha-helical conformation. PrP^{C} is mostly found on the cell membrane of neurons and at lower levels in others tissue types. The disease-associated isoform PrP^{Sc} is insoluble in detergent and relatively resistant to protease digestion; therefore, proteinase K treatment is commonly applied to distinguish PrP^{Sc} from PrP^{C} (*2*). The prion hypothesis proposes that PrP^{Sc} seeds the protein misfolding process, thereby converting alpha-helix-rich PrP^{C} into the amyloid form (β-sheet-rich) PrP^{Sc} (*3*). TSE diseases are associated with misfolded prion protein (PrP^{Sc}) deposition in the CNS, lymphoid tissues and peripheral nervous system (PNS) during disease progression until the end stage. PrP^{Sc} is mainly found within the brain when cattle, sheep, human, deer, experimental rodents or non-human primates are afflicted with TSE diseases but PrP^{Sc} can also be observed in peripheral tissues for some strains (classical scrapie, vCJD and CWD) (*4-6*). Animals showing clinical symptoms and neurodegenerative pathology normally also have PrP^{Sc} deposition. Therefore, proteinase K (PK) resistant PrP^{Sc} is commonly used as a TSE disease marker.

Classical scrapie mainly affects sheep, and occasionally found in goats. The disease is found in many European countries and worldwide (with the exception of New Zealand and Australia, which are considered free from classical scrapie). Characteristics of classical scrapie include the long incubation period of the disease (between two and five years) and the fact that it can be transmitted vertically from ewes to lambs at birth; later studies have proven that it can also be

transmitted through milk, saliva and environmental routes (*7*). Since 1967, deer in North America have been afflicted by an epidemic of CWD that has threatened both wild and captive farmed deer. The infectious agent of CWD (PrP^{Sc}; also termed PrP^{CWD}) can be shed easily into the environment through excreta such as urine, faeces and saliva (*8*). This has made CWD a highly transmissible disease among deer and other cervid species such as elk and moose. Deer can easily acquire the CWD agent through contact, grazing and drinking of contaminated water sources. Data from human transgenic mice models suggest that CWD does not have zoonotic potential (*9*).

BSE (commonly known as "mad cow disease") was first diagnosed in the UK in 1986, and later became an epidemic throughout Western Europe and worldwide. The cause of BSE is believed due to meat and bone meal (MBM) feeding practices used at the time – this resulted in animals ingesting potentially contaminated material (*10*). The practice has been banned throughout European countries but the origin of BSE remains a debate in the field. Suggested theories for the causal agent of BSE include sporadic BSE, genetic BSE and classical scrapie (*11*). Since the ban of MBM in the farming industry, there has been a sharp decline in BSE cases. Although BSE cases have been reduced after the ban of MBM, BSE has not been completely eradicated – there are still random cases of BSE discovered in cattle from year to year (*11*). BSE is a lethal disease that has been found to cross the species barrier, thereby contributing to interspecies infection through contaminated food sources. Experimental transmission of BSE to sheep, non-human primate, rodents and deer has also occurred (*10*). Natural transmission of BSE to humans causes vCJD; in cat species it causes feline spongiform encephalopathy (FSE) in captive exotic ungulates in the UK, such as nyala and greater kudu (*12*), it has caused exotic ungulate encephalopathy (EUE). BSE has also been transmitted experimentally into rodents, small ruminants, non-human primates and pigs (*13, 14*). Although there is no evidence of naturally occurring sheep BSE, sheep are known to be susceptible to experimental BSE infection (*7*). Furthermore, some cases of naturally occurring goat BSE have been reported in the UK and France (*13*).

The human forms of TSE diseases are categorised into sporadic, inherited and acquired diseases. These include – but are not limited to – kuru, sCJD, Gerstmann–Sträussler–Scheinker syndrome, fatal familial insomnia (FFI) and vCJD (*15*). A sporadic form of CJD accounts for most of the CJD cases (80%) – the yearly incidence rate of sporadic CJD is around one case per million people (*16*). Acquired forms of TSE diseases include kuru, iatrogenic CJD and vCJD. These are caused by

ingestion and/or accidental introduction of infectious agents during surgical procedures. Kuru was caused by the ritual consumption of prion contaminated (kuru-infected) human material (*6*). Iatrogenic CJD has been transmitted by CJD-contaminated surgery equipment and/or procedures such as dura mater grafts and pituitary hormone treatments (*17*). VCJD was first described in 1996 and it was suggested that exposure to BSE-contaminated food sources was the cause of the disease (*5*). Experiments have shown that BSE and vCJD are caused by the same strain of infectious agents (*18, 19*). VCJD normally has a long clinical course (median of 13 - 14 months) and mostly affects young adults. FFI, GSS and familial CJD are prion diseases responsible for 15% of CJD cases and are caused by genetic mutations in the *PRNP* gene coding for PrP protein (*20*).

Genetic influence on susceptibility and pathogenesis of TSE diseases

The gene coding for the PrP protein, termed *PRNP* (prn-p), is widely studied in relation to TSEs. Sheep are the natural host to classical scrapie, and the *PRNP* gene in sheep is highly polymorphic, particularly at positions 136, 141, 154, 168 and 171 (*21*). Single nucleotide polymorphism within *PRNP* are commonly linked to susceptibility and variation in the incubation period in TSEs (*21*). *PRNP* polymorphisms at position 136, 154 and 171 (homozygote ARQ) are the archetype or wild type at these positions (*21*). Classical scrapie studies have indicated that the sheep *PRNP* genotype, in particular at codons 136 and 171, can influence susceptibility and other disease phenotypes such as survival times, clinical signs, vacuolation profiles and histology patterns in the brain (*22*). Besides that, *PRNP* homozygous at codon 141 (L_{141}) has been associated with the shortest disease incubation in the cases of sheep TSEs (BSE and scrapie) compared with homozygote F_{141} and heterozygote LF_{141} (*22, 23*). The polymorphisms in *PRNP* have been linked to the efficiency of PrP^{Sc} seeding PrP^{C} conversion to the misfolded form and/or influencing susceptibility or resistance to TSEs in *in vitro* conversion studies (*24, 25*). The most susceptible genotype in the case of classical scrapie is homozygote $V_{136}R_{154}Q_{171,}$ while $A_{136}R_{154}R_{171}$ and $A_{136}H_{154}Q_{171}$ are genotypes linked to resistance to classical scrapie (*22, 26*). In addition to susceptibility, the incubation times have been studied intensively in sheep models. Sheep with homozygote $A_{136}R_{154}Q_{171}$ have been shown to have the shortest incubation periods when experimentally infected with BSE (*13*). Hence, sheep with this genotype have been employed for studying BSE transmission in a large animal model (*27*). The effect of genotype in TSEs have been proven to relate to peripheral pathogenesis, which plays a major role during the natural occurrence of TSE diseases (*28, 29*).

Peripheral pathogenesis of TSE diseases

The most common natural route of TSE infection is via oral uptake of the infectious agent (PrP^{Sc}), from the environment or contaminated food sources (*30*, *31*). In addition to the major sites in the CNS and PNS, PrP^{Sc} deposition may also be observed in peripheral tissues such as gut-associated lymphoid tissues (GALT), spleen, skin, mammary glands, gastrointestinal tract and palatine tonsil (*32*). Both classical scrapie and BSE in sheep are associated with widespread PrP^{Sc} deposition in the central nervous system, lymphoid tissues and other organs (*33*, *34*). Mice and sheep models have already proven that classical scrapie and BSE infection can show peripheral pathogenesis resembling that of human vCJD (*29*, *35*, *36*). Studies of classical scrapie and BSE-infected mice showed that PrP^{Sc} deposition was found in the Peyer's patches and mesenteric lymph nodes during the early stage of infection before progressing into the spleen and non-GALT tissues via the blood (*37*). Tissues from BSE-infected mice showed PrP^{Sc} disseminated in the GALT, spinal cord, enteric nervous system (ENS) and brain (*38*, *39*). Experimental sheep-BSE showed a similar widespread distribution of PrP^{Sc}, with accumulation being found in the lymphoid system, digestive tract, some components of the peripheral nervous system (PNS) and in the CNS (*34*). The PrP^{Sc} deposition in sheep was greatly different from classical BSE in cattle, for which there is limited evidence of PrP^{Sc} manifestation in the peripheral organs (*40*). Experimental models of macaque-adapted BSE showed that PK-resistant PrP^{Sc} is also found in similar organs to the sheep and mice studies (*14*).

During oral infection, the palatine tonsil is frequently found with deposition of PrP^{Sc} in classical scrapie, experimental BSE and vCJD patients, but the main sites for PrP^{Sc} replication are GALT and lymphoid tissues (such as Peyer's patches, mesenteric lymph nodes (MLN), palatine tonsil, prescapular lymph nodes (PSLN) and spleen) (*34*, *36*, *39*). Various cell types within GALT are associated with direct and indirect uptake, transportation, degradation and replication of PrP^{Sc} in an animal (*41*, *42*). **Figure 1** shows the standard sequence of particular TSE disease progression (peripheral pathogenesis) during the oral route of infection before reaching the CNS. In the Peyer's patches, microfold cells (M cells) and enterocytes are responsible for PrP^{Sc} transportation (transcytosis) from the lumen (gut) to nearby dendritic cells (*43*). PrP^{Sc} then is consumed by macrophages or dendritic cells before capture by the follicular dendritic cells (FDC) localised in the B cell follicles of GALT (*44*, *45*). The FDC plays an important role by acting as the reservoir for PrP^{Sc} accumulation in GALT. FDCs that require lymphotoxin β signals from B cells to maintain their

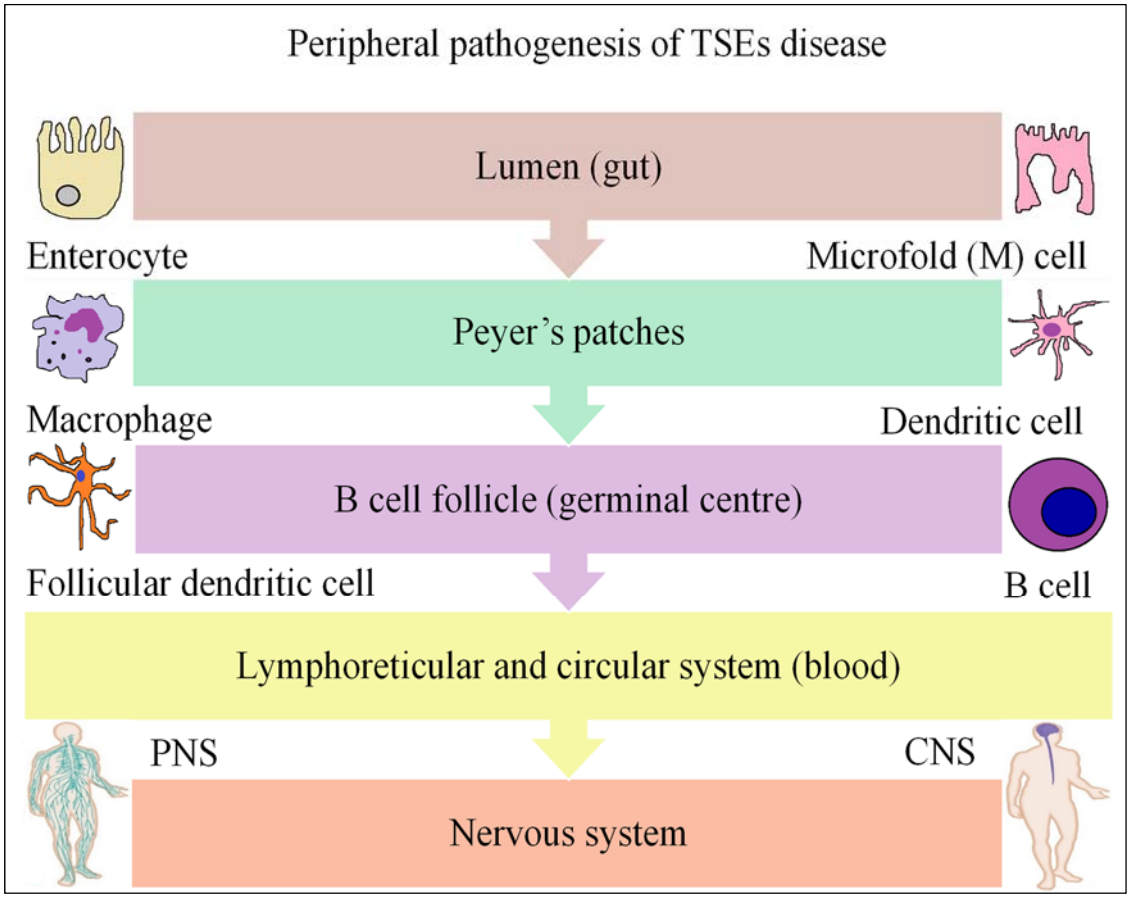

Figure 1: Peripheral pathogenesis of TSE disease during oral infection.
The diagram shows a sequence of events which describe the naturally occurring TSEs (such as BSE, scrapie and CWD) disease. PrP^{Sc} is transmitted through the oral infection route when a host consumes the contaminated food source. M cells and enterocyte are the cells that uptake PrP^{Sc} from the gut through a transcytosis process before macrophages and dendritic cells capture the 'foreign' antigen in the Peyer's patches. FDCs that reside at B cell follicles of germinal centre in Peyer's patches capture the PrP^{Sc}. PrP^{Sc} then adheres to B cells during FDC maturation, which is initiated by B cells. PrP^{Sc} then spreads throughout GALT, the lymphoreticular system and the blood via B cells, T cells, monocytes and blood components. An event termed neuroinvasion occurs when PrP^{Sc} transmits to the PNS and reaches the CNS.

differentiated state will interact with B cells during the process of maturation (*46*). B cells indirectly become the carrier of PrP^{Sc} when probing the surface of FDCs for antigen. Thus, B cells carrying PrP^{Sc} are involved in the blood transmission route of TSE disease (*47*). Impairment of FDCs has already been shown to delay scrapie progression in mice models when – lymphotoxin β receptor was blocked by using a lymphotoxin β receptor-immunoglobulin (LTβR-Ig) fusion protein (*48*). In the study, intraperitoneal injection of LTβR-Ig three days before the oral inoculation of scrapie resulted in no accumulation of PrP^{Sc} in the spleen, Peyer's patches and MLN, which substantially reduced the disease susceptibility in the mice model (*48*). Another

study with sphingosine 1-phosphate receptor (S1PR1) showed to control the exit of B cells, during their recirculation between lymphoid tissues (*49*). An experiment demonstrated that white-tailed deer medulla, palatine tonsil and retropharyngeal lymph nodes were found to have PrP^{Sc} deposition after receiving B cells from CWD-infected deer (*47*) showing they are capable of carrying and transmitting infection. Besides B cells, tingible body macrophages (TBMs) are resident around B cell follicles and act as scavengers to phagocytose apoptotic B cells carrying foreign antigen (in this case is PrP^{Sc}) (*50*). TBMs are macrophages implicated in PrP^{Sc} deposition within the germinal centres (GC – sites where mature B cells proliferate, differentiate and respond to an infection) of lymphoid follicles (*51*). PrP^{Sc} deposition on TMBs can be detected as early as six months after orally challenging sheep with BSE (*52*).

The long incubation periods in TSE diseases may be partially related to the efficiency of the peripheral route of infection and PrP^{Sc} replication. Early accumulation of PrP^{Sc} is found in draining lymph nodes of sheep orally infected with BSE, which suggests that these sites are crucial for PrP^{Sc} replication (*38*). The lymphoreticular system (LRS) would then play an important role during the amplification and distribution of PrP^{Sc} in the whole animal (*28, 37*). Hence, lymphoid tissues, especially GALT, have been suggested as the reservoirs for PrP^{Sc} replication to take place (*30, 37*). Since the LRS is interconnected, when PrP^{Sc} is harboured at the GC it will be spread by B cells during the disease pathogenesis (*53*). Cells with cytoplasmic PrP^{Sc} granules were observed migrating through lymph nodes, representing a lymphatic/vascular dissemination pathway (*30*). In the LRS, the spleen is another organ that serves as the reservoir for PrP^{Sc}; therefore, it is closely linked to infectivity in TSE (*54*). The evidence of PrP^{Sc} spread through cell migration within GALT and others LRS such as the spleen or the mediastinal lymph node has been shown by immunolabelling in classical scrapie-infected sheep (*30*).

The term neuroinvasion is used to describe the development of prion disease from LRS to PNS via lymphoid tissues and eventually leading to accumulation in the CNS (*28, 29, 33, 42*). Neuroinvasion has been demonstrated by tracking PrP^{Sc} channelling from the LRS to the CNS either via nerve fibres of the sympathetic nervous system or the parasympathetic nervous system (*42*). Neuroinvasion starts from the parasympathetic nerve fibres of GALT (*33*). The first peripheral nervous system (PNS) site of PrP^{Sc} accumulation was shown to be the post-ganglionic parasympathetic and/or sympathetic motor neurons in the gastrointestinal wall (*55*). PrP^{Sc} also was followed through to the enteric nervous system, spinal cord and was first shown in the dorsal

motor nucleus of the vagus (DMNV) nerve in the brain (*55*). Hence, the long incubation time of prion disease can be explained by the inefficiency of PrP^{Sc} replication in the lymphoid tissues, and the requirement for progression to the PNS (particularly sensory and autonomic ganglia) before reaching the CNS (*56*).

Blood route of TSEs infectivity

Besides neuroinvasion, the haematogenous route of infection is known for its complexity during TSE pathogenesis. Although PrP^{Sc} is present in most TSE diseases, the deposition of PrP^{Sc} does not always correlate to infectivity of TSE diseases – one research group showed that over half of BSE-infected mice were without PrP^{Sc} deposition (*57*). Thus, investigation of TSE infectivity via bioassay is vital. Scrapie infectivity and PrP^{Sc} are spread by cells that express CD21[a], which is involved in the activation and maturation of B cells and FDC (*58, 59*). In rodent models, studies have shown that leucocytes are associated with infectivity in the blood (*60, 61*). Involvement of whole blood, buffy coat, platelet and plasma with TSE infectivity have been illustrated previously through the intravenous route of transmission in ruminants (*62, 63*). Experiments with blood components have established that a minimum of 10^5 WBC can transmit scrapie (*64*). More specifically TSE infectivity has been found to correlate with B cells (*47, 49*), T cells (*65*), peripheral blood monocytes (PBMC) (*66*), platelets (*58, 67*), coagulation factors (*68*) and plasma (*62*). Evidence of PrP^{Sc} has been found within CD21 positive B cells, T cells and monocytes in the peripheral blood mononuclear cell (PBMC) and platelet in both classical scrapie and CWD-infected animals, suggesting that blood is one of the routes of TSE infectivity and PrP^{Sc} accumulation in infected animals (*47, 58, 65*).

Numerous sheep studies have shown that both BSE and classical scrapie can easily be transmitted via the blood transfusion route (*69*). The intravenous challenge with PG127[b] scrapie showed that whole blood was infectious when collected at 50% (between 160 and 170 days post-inoculation, dpi) of the disease incubation period (231 dpi ± 15, SD) (*70*). Sheep-BSE showed that blood components were infectious around one-fourth (25%) of the incubation time at 939 dpi ± 316, SD (*23*), which was similar to the experiment with classical scrapie (28% - 80%) at 729 dpi ± 99, SD (*69*). Collectively, studies have shown that TSEs can be transmitted through the haematogenous

[a] Cluster of differentiation 21 is also known to be involved in the innate immune response.
[b] A classical scrapie isolate derived from experimentally infected VRQ/VRQ sheep.

route in rodents (*71, 72*), small ruminants (*58, 69, 72*), cervids (*47, 63*) and non-human primates (*73*). To confirm positive PrP^{Sc} deposition in the blood samples, some studies have employed *in vitro* amplification assays, such as the protein misfolding cyclic amplification (PMCA) method, in order to detect PrP^{Sc} in vCJD-infected non-human primates (*73*).

Variant Creutzfeldt-Jacob disease (vCJD) first appeared after the BSE epidemic and later confirmed to be originated from the BSE agent (*18*). To date, 231 cases of vCJD have been diagnosed worldwide, and the UK has had a high infection rate of 178 cases during the peak of the BSE epidemic (*74*). Most vCJD cases were caused by primary infection through consumption of BSE-contaminated beef products; out of the 178 cases, four secondary cases were found in relation to blood transfusion (*75*). Recipients transfused with contaminated blood components from vCJD donors have been found to have PrP^{Sc} deposition in either peripheral tissues and/or in the brain (*75*). The deposition of PrP^{Sc} in non-neuronal tissues (including blood) has raised the possibility that there could be a risk of acquiring iatrogenic TSEs through surgery procedures (*76*) or by transmission of blood components (*77*). So far, 67 recipients are known to have been transfused with vCJD-contaminated blood products (*75*). None of the 34 deceased patients carried out autopsy examination for TSEs, which indicated unknown status for vCJD (*75*). Three recipients who were transfused with vCJD-contaminated blood components were diagnosed with vCJD, with another recipient confirmed as having PrP^{Sc} deposition in the spleen after post-mortem diagnosis. As of writing, the remaining 14 recipients are still alive without any clinical symptoms (*74, 75*). The level of PrP^{Sc} might change during the incubation of the disease (*78*), meaning that it is hard for the current healthcare system to diagnose vCJD in blood samples. Therefore, a large animal model was required to assess the pathogenesis of BSE transmission by looking at the infectivity and the PrP^{Sc} distribution.

Blood transfusion models for animal TSEs

VCJD has been linked to the blood route transmission (*79, 80*) in a similar manner to experimental sheep models (*62, 69*). Neuropathological examination of the necropsy tissues from vCJD patients demonstrated the same pattern of PrP^{Sc} deposition in tissues as was the case in BSE-infected sheep (*34, 81*). The similarities of peripheral pathogenesis between sheep (BSE and classical scrapie) (*27, 38*) and humans (*82*) indicates that the sheep model is the best available model for vCJD study. Sheep have a similar blood volume to a human adult, and the blood component composition matches the standard of the NHS blood and transplant (NHSBT) institution (*69, 83*). Hence, sheep

orally infected with BSE offer an ideal model to study transfusion-associated blood-borne vCJD. This is further supported by the later finding of similar setting for blood components such as whole blood, buffy coat, red cell concentrates, plasma, platelet and their leuco-depleted derivatives (*62*). Experimental transfusion of scrapie-infected blood components between sheep (*72*) has shown that white blood cells (WBC) and whole blood are highly infectious, as was the case in the sheep BSE study (*62*). When scrapie-infected WBC were fixed with 2% paraformaldehyde, it affected the transmission rate in sheep and reduced the infectivity of WBC in mice (*72*). Scrapie-infected blood transfusion in sheep also confirmed that platelet-rich blood components are infectious, which was similar to the sheep-BSE study (*58, 62*). Studies of CWD-infected white tail deer showed that whole blood and buffy coat are extremely infectious, followed by platelet components (*47*). However, when an animal is transfused with cell-free plasma, no infectivity is observed, which indicates that white blood cells and platelets are essential in TSE infectivity (*47*). The sensitivity and specificity of methods to detect TSE infectivity and/or PrP^{Sc} in body fluids, especially blood, have been a challenge in TSE research.

Diagnosis of TSE diseases

Bioassay has been used frequently to detect the infectivity of TSE diseases; this includes rodents (*71*), non-human primates (*14*), sheep (*69*) and/or cell-based assays (*84*). Bioassays can be time-consuming and costly; therefore, diagnosis of TSE diseases has used the detection of PrP^{Sc} as an alternative. The gold standard of PrP^{Sc} detection is using an immunoassay such as Western blot, immunohistochemistry or ELISA, in which PrP^{Sc} is detected with anti-PrP antibodies (*52, 85, 86*). The main obstacles in the prion field are the issues of biopsy samples (which are normally invasive and labour intensive to obtain), the sensitivity of the immunoassay and the quantification of PrP^{Sc} from lymphoid tissues and body fluid. The PrP^{Sc} levels in lymphoid tissues such as the spleen and palatine tonsil have been found to be 2 to 3 logs lower than in the brain (*82, 87*). Some examples of diagnostic assays that detecting the PrP^{Sc} by different approaches which included using anti-PrP antibodies to detect PK-resistant PrP^{Sc} (*88, 89*), the denaturation method to measure the different ratio of epitope differences between PrP^{C} and PrP^{Sc} (*90, 91*), the affinity of the PrP protein to metals and/or ligands (*92-95*), and the PrP protein as the substrate for detection of PrP^{Sc} from body fluids through *in vitro* amplification assays (*96, 97*).

The ELISA detection method involves adsorption of an anti-PrP antibody to the bottom of the microplate wells prior to capturing the target PrP^{Sc} with the second detector anti-PrP antibody.

The ELISA format has used an anti-PrP antibody coupled directly or indirectly to an enzyme that will produce a chromogenic signal that can be measured by a plate reader. The nature of PrP^{C} sensitivity to proteinase K digestion is a principle that has been applied in both WB and ELISA to detect PK-resistant PrP^{Sc} (*98*). Studies of the medulla oblongata from asymptomatic sheep infected with classical scrapie or BSE have shown that the Bio-Rad TeSeE™ sheep/goat kit and the IDEXX HerdCheck BSE-scrapie antigen test kit are reproducible and sufficiently sensitive to detect scrapie in sheep (*99*).

Since body fluids such as cerebrospinal fluid (CSF) and blood have low levels of PrP^{Sc}, immunoprecipitation (IP) has been applied to enhance PrP^{Sc} detection in the body fluids down to the picogram level (*100*). Furthermore, the PrP protein has the property of being able to bind to metal ions such as stainless steel, also providing the possibility to capture PrP^{Sc} using a solid-state capture matrix (*92, 101*). The direct detection assay used a solid-state binding matrix to detect PrP^{Sc} down to 10^{-10} dilution level in the whole blood samples spiked with vCJD-infected brain (*92, 102*). Similarly, an IP method that used magnetic beads to pull out PrP^{Sc} has been combined with ELISA, enabling the detection of 2×10^{4} (units/ml) lethal dose (LD_{50}) equivalent to 700 femtograms of PrP^{Sc} in 10µl of whole blood (*103*). An alternative IP method using the plasminogen-based assay showed the full capturing efficiency in the end-point dilutions to 10^{-8} level in an experiment of plasma spiked with human vCJD (*94*). Besides using IP methods, a more consistent way to detect low level of PrP^{Sc} is by using the *in vitro* amplification assays such as protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC) (*104, 105*).

<u>Protein misfolding cyclic amplification (PMCA)</u>

The principle behind the *in vitro* amplification assay is the prion hypothesis – PrP^{Sc} is used as a seed to induce protein misfolding and amplification of substrates (PrP^{C} or recombinant PrP protein) to form the misfolded amyloid form of PrP^{Sc} (*106*). PMCA technology has been developed for pre-clinical detection of PrP^{Sc}. The assay mimics the nature of the PrP^{Sc} replication process by using sonication to break down aggregates in an excess of substrate (PrP^{C}) to accelerate the PrP^{Sc} replication artificially (*96*). Brain homogenate serves as the substrate in PMCA since it is rich in PrP^{C}. PMCA was originally developed to detect 263K (hamster-adapted scrapie), with the improvement of the technique PMCA can now detect PrP^{Sc} in TSE-infected rodent, cattle, sheep, human and deer brain (*107-109*). In PMCA, disassociation of PrP^{Sc} aggregates by sonication will create more monomers that serve as the templates for conversion (*96, 110*). An adapted form of

serial PMCA (sPMCA) replenishes PrP^{C} in continuous cycles (one day is considered as one cycle) to support the amplification processing until PrP^{Sc} reaches the detectable level (by WB) (*111*). PMCA has been used to detect PrP^{Sc} from body fluids (blood, urine, CSF), but this approach has the challenge of inhibiting factors that can interfere in PMCA (*60, 112*). Nevertheless, published reports have shown that PrP^{Sc} can be detected in 10^{6} cells (~0.5ml) of sheep blood (*113*), in scrapie-affected hamster urine as low as 2.0×10^{-16} g/ml (*114*), in preclinical scrapie-infected hamster as early as 20 dpi (*115*), and in CSF of CWD-infected deer (*116*). PMCA has revealed that whole blood and white blood cells (WBC) are highly infectious blood components (*65, 67, 113*). SPMCA is a time-consuming process that takes a minimum of three days (three cycles). In some cases, false positive (spontaneous PrP^{Sc}) results can occur during the multiple rounds of amplification (*113, 117*). Besides being used as a detection tool, PMCA also can be utilised to study interspecies transmission events such as accessing the zoonotic potential of sheep-BSE (*118*). Despite the success of PMCA in detecting PrP^{Sc} in blood, the limitation of PMCA in terms of assay detection time and the infectious state of the product makes RT-QuIC an ideal alternative.

Real-time quaking-induced conversion (RT-QuIC)

RT-QuIC is designed to amplify misfolded PrP^{Sc} (seed) aggregation *in vitro*, which is enabled by using bacterially generated recombinant PrP (rPrP) protein as the substrate (*119*). The basic mechanism of RT-QuIC is based on the seeded conversion reaction of rPrP, involving periodic intervals of shaking and resting in a plate reader. This process will initiate the conversion of rPrP into misfolded PrP^{Sc} amyloid that is rich in β-sheets (*120*). **Figure 2** shows the use of thioflavin T (ThT) as a dye to detect PrP^{Sc} aggregates in RT-QuIC. By binding to β-sheet rich structures,

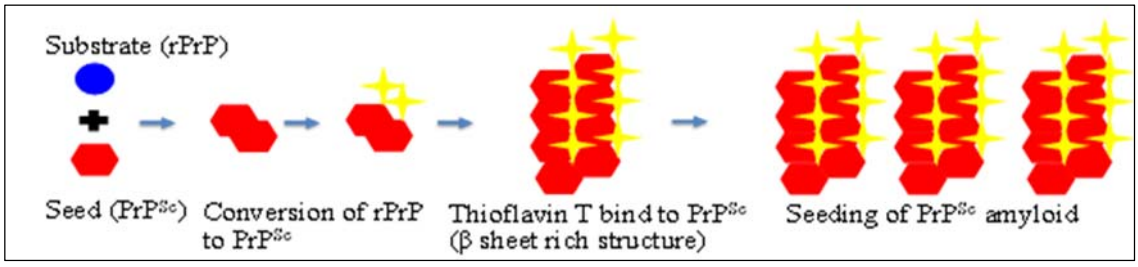

Figure 2: Mechanism of real-time quaking induced conversion (RT-QuIC).
RT-QuIC used seed (PrP^{Sc}) to induce the conversion of substrate (rPrP) from the normal form into the misfolded β-sheet rich (amyloid) form of PrP^{Sc}. The aggregation of seed will elongate in aggregated fibril form called amyloid. Amyloid seeding is the process where more and more substrate is converted into amyloid seeds during shaking and resting interval in the reaction buffer. Thioflavin T (ThT) dye that binds to the amyloid structure will undergo 450nm excitation and 480nm emission in a microplate reader (BMG Labtech, POLARstar) to give relative fluorescence units. Hence, the amyloid seeding activity can be monitored in real-time.

fluorescence signals are emitted by ThT and these are measured in real time using a high-throughput fluorescence plate reader (*121*). The elongation of PrP^{Sc} aggregation (fibril form) or amyloid also described by the seeding activity as an increase in fluorescence signal until it plateaus at the horizontal asymptote (top) when the aggregation process stabilises (illustrated as a sigmoidal curve). The advanced in QuIC protocol has proven to be reproducible for amplifying PrP^{Sc} from 0.12 femtogram of 263K brain homogenate in less than two days (*122*). The hamster rPrP substrate was first used to study the principle of the protein misfolding process for TSE diseases such as 263K, classical scrapie and sCJD (*123*). Subsequently, it was assessed whether using rPrP such as ham-sheep chimeric, truncated hamster, human, mouse or bank vole would improve the RT-QuIC sensitivity of various TSE strains (*124, 125*). Besides that, RT-QuIC has proven to be highly sensitive for detecting PrP^{Sc} from samples such as CNS, CSF, lymphoid tissues and blood components (*116, 126, 127*). As an example, sCJD subtype brain homogenates have been reported to seed rPrP substrate at the femtogram ($1x10^{-15}$ g) level of PrP^{Sc} (*112*). It has shown to have a sensitivity of 87% and specificity of 100% for the diagnosis of sCJD patient CSF samples (*128*).

Immunoprecipitation (IP) has been applied to improve the sensitivity of PrP^{Sc} detection in blood plasma for the RT-QuIC – this process can reduce the interference of inhibitors during the amyloid seeding process of PrP^{Sc} (*129*). CWD and hamster-adapted TME (PrP^{Sc}) in the blood have proven to be detectable when the sodium phosphotungstic acid (NaPTA) precipitation step was applied before RT-QuIC (*130*). Enhanced QuIC (eQuIC) is another method that employs immunoaffinity beads coupled with the 15B3 (anti-PrP^{Sc}) antibody. This method has tremendously improved the sensitivity of detecting PrP^{Sc} in the plasma of classical scrapie-infected hamsters (*129*). Following on from this success, the eQuIC protocol has been adapted to use hamster-sheep chimeric rPrP as the substrate. Such a change was used to push the boundary of detection sensitivity (to attogram level) and to shorten the detection time (28 hrs) for human blood samples spiked with vCJD brain (*129*). Another advantage of RT-QuIC is that the end product (PrP^{Sc}) are non-infectious, thereby creating a relatively safe diagnostic platform for large-scale screening (*128*). Recently, improvements of RT-QuIC by increasing the temperature and/or shaking speed have successfully reduced the amplification time while still maintaining the specificity (*131*).

The seeding activity has been revealed to differ according to the level of PrP^{Sc} (*124*). RT-QuIC has been proven as measurable by using the end-point dilution method, where the seeding dilution that gives half of PrP^{Sc} is defined as the seeding dose (SD_{50}) per unit of the sample (*132*). Measurement of SD_{50} from 263K-infected hamster brain showed that the seeding activity could be estimated within a shorter time frame; furthermore, it required 25 times fewer samples than bioassays to reach the sensitivity (*132*). The PrP^{Sc} seeding rate demonstrated the signature pattern of lag phase resembling that of the RT-PCR reaction (*133*). Nucleation is used to describe the process that assembles protein monomers (rPrP) to form the precursor structure (*134*). Thus, PrP^{Sc} has been proposed to constitute stable fibrils at the initial stage through to the nucleation time (lag phase), likewise known as the time to reach threshold (C_t) (*135*). Bioassays have the advantage of detecting infectivity but are generally expensive and time-consuming (*68*); the quantitative study of PrP^{Sc} seeding rate (SD_{50}) by using the end-point titration method has proven analogous to the infectivity titre measured by LD_{50} (*135*), thus, can be used as an alternative to measure infectivity.

Project background

The ovine BSE experiment showed that incubation period is related to *PRNP* genotype at codon 141 (*23*), but the underlying mechanism that contributes to the differences in incubation is inconclusive. Since no major differences in PrP^{Sc} deposition were found in the terminal medulla, the PrP^{Sc} levels in the peripheral tissues, especially lymphoid tissues and blood should be investigated. **Figure 3** shows the histopathology data (unpublished) indicating that the donor sheep with homozygote L_{141} genotype (transmitters) had transmitted BSE to the recipients – all of their lymphoid tissues were positive for PrP^{Sc} deposition. On the other hand, BSE-positive donors that did not transmit the disease (non-transmitters) were mostly from heterozygote LF_{141} genotype, with only 58% of their lymphoid tissues showing PrP^{Sc} deposition. However, variation in the infection rate was observed among the recipient sheep implying there might be differences in infectious load.

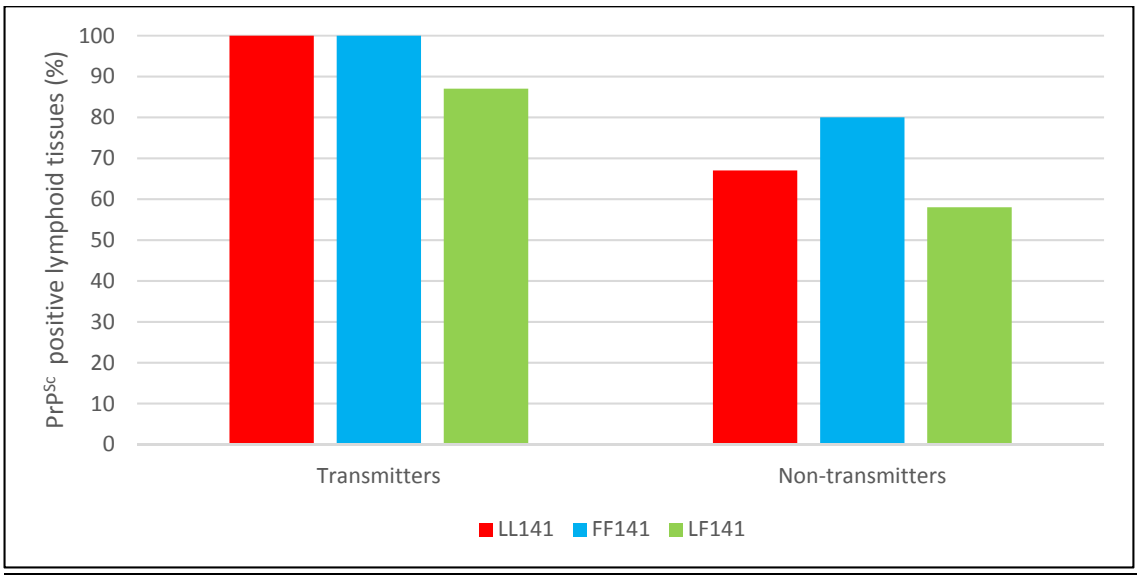

Figure 3: Percentages of PrP^{Sc}-positive lymphoid tissues in ovine BSE-infected transmitters and non-transmitters sheep.

The graph shows ovine BSE-positive donors (sheep) categorised either as transmitters or as non-transmitters depending upon whether they transmitted ovine BSE to the recipients. The colour coding is used to define the codon 141 genotype and the graph shows the percentages of lymphoid tissues that were PrP^{Sc}-positive (immunohistochemistry results).

Project hypothesis and objectives

Analysis of the sheep-BSE (blood transfusion study) results showed that the incubation time for donor sheep was associated with *PRNP* genotype variation at codon 141 (*23*). *PRNP* codon LF_{141} heterozygous animals were found to have fewer lymphoid tissues positive for PrP^{Sc} deposition compared to homozygotes L_{141} or F_{141} (unpublished data). Immunohistochemistry results suggested that there was a variation in the level of staining in lymphoid tissues. Since PrP^{Sc} has always been used as the marker for TSE disease, studies to quantify PrP^{Sc} in lymphoid tissues are therefore required to understand if the single polymorphism (codon 141) can influence PrP^{Sc} levels (deposition) in lymphoid tissues. A pilot study was conducted to determine whether the levels of PrP^{Sc} in the lymphoid tissues vary depending on the codon 141 genotype. Since no published method for quantifying ovine BSE using ELISA was available, the initial part of the project was to adapt the TeSeE sheep/goat ELISA for quantitative study – the TeSeE sheep/goat kit is an EU approved diagnostic kit for the detection of BSE in ruminant tissues. Although the peripheral pathogenesis has been widely studied, the correlation between ovine BSE infectivity and PrP^{Sc} level remains unknown. Therefore, we proposed to develop an established method RT-QuIC to estimate the level of PrP^{Sc} and/or SD_{50} that can be used to correlate with the infectivity in tissue at the later stage of the project. After optimising on RT-QuIC at the end of this pilot study, the technique can be used in future work to detect relative PrP^{Sc} level and correlate to infectivity in blood component from sheep BSE blood transfusion study.

Objectives

a) Develop TeSeE™ sheep/goat kit and RT-QuIC for quantitative study.
b) Analyse quantitative ELISA results by comparing the relative PrP^{Sc} levels among codon 141 genotypes.
c) Determine the detection limit (minimal brain equivalent to PrP^{Sc} detection) among ELISA, WB and RT- QuIC.

Project designs

a) Initially, a known concentration of truncated ovine $A_{136}R_{154}Q_{171}$ rPrP protein was used as the calibrator (standard) to estimate the relative levels of PrP^{Sc} (rPrP-to-total protein) in the brain and lymphoid tissues. Quantitative ELISA was also validated for precision and reproducibility by calculating intra- and inter-assay coefficient of variation (CV, %). RT-QuIC was optimised to determine the suitable substrate (rPrP) and condition (temperature) for initiate amyloid seeding specific for ovine BSE. The amyloid seeding rate was calculated on end-point dilution of BSE-infected brain based on a previously published method (*135*).

b) At least five samples of each codon 141 genotypes were used for the measurement of relative PrP^{Sc} levels. The rPrP equivalent of PrP^{Sc} was calculated with their total protein to get the relative PrP^{Sc} (ng/mg). The results of relative PrP^{Sc} were used to test the hypothesis on the contribution of genotype variation to PrP^{Sc} deposition in lymphoid tissues.

c) Two sets of brain dilution series (two-fold and ten-fold) were prepared for comparison of the detection limit among ELISA, WB and RT-QuIC assays. The lowest PrP^{Sc} detectable brain equivalent was determined in each assay.

Methods and materials

Experimental animals and post-mortem sample collection

New Zealand-derived Cheviot sheep obtained from DEFRA scrapie-free flock were selected for the homozygous $A_{136}R_{154}P_{168}Q_{171}$ *PRNP* genotype, but codon 141 was polymorphic. More details of sheep and experimental design were described in (*62*). Experiments were approved by Animal welfare and Ethical Review Committees[c] and conducted under the authority of Home Office Project Licences, according to Animals (Scientific Procedures) Act 1986. Donor sheep were orally challenged with five grams of BSE-infected bovine brain homogenate[d] (n = 39), or with five grams of uninfected bovine brain homogenate as negative controls (n = 9). Various blood components were taken from donors at the ten-month time point to transfuse into the recipient group. The following components were made from donor sheep and transfused to recipient sheep: whole blood (WB, n = 8), buffy coat (BC, n = 37), red cell concentrates (RCC, n = 37), plasma (PLS, n = 38), and platelet concentrates (PLT, n = 37). At the same time, uninfected whole blood from mock-infected control donors was transfused into nine recipient sheep. Separate groups of recipient sheep were transfused with leucodepleted blood components as described in (*62*). All sheep were clinically monitored until a terminal end-point – either display of TSE clinical signs[e] or culling for welfare reasons[f]. During the post mortem, samples of brain, tonsil, spleen, distal ileal Peyer's patch, mesenteric lymph node (MLN) and prescapular lymph node (PSLN) were collected, and fixed in neutral buffered formalin or frozen at -80°C. Western blot (described as follows) and immunohistochemistry were carried out using any two types of monoclonal anti- PrP antibodies (ROS-BC6, ROS-BH1 or ROS-IH9) on each assay to screen the brain and lymphoid tissues for PrP^{Sc} deposition compared with uninfected control.

Preparation of tissue homogenates

Frozen tissues were dissected and homogenates were prepared in a microbiological safety cabinet class II (MSC II) within a derogated Containment Level 3 laboratory. The outer container was sterilised with 70% (v/v) ethanol before and after dissection steps. The brain was thawed at 4°C for 30 minutes, followed by one hour thawing at room temperature (in MSC II cabinet).

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[c] Previously Institute for Animal Health currently based at The Roslin Institute under the University of Edinburgh.

[d] Obtained from the Animal and Plant Health Agency Biological Archive Group, Weybridge.

[e] Either pruritus (continuous scratching of wool) and/or ataxia (abnormal gait).

[f] Such as weight-loss, not feeding or sickness caused by non-TSE agent.

Lymphoid tissues (MLN, PSLN and spleen) were placed in a -20^{0}C freezer for at least one hour until dissection steps. From the brain, the medulla oblongata located caudal to the pons was dissected and weighed in order to prepare homogenates in PBS using tissue homogenizer (Omni, TH220). A volume of PBS equal to ten times the tissue weight was added prior to homogenisation until no tissue pieces were observed in the homogenate (normally up to two minutes of homogenisation depends on the tissue types). Lymphoid tissues were dissected from the anterior, posterior and middle region of frozen tissues to pool together the tissues for homogenisation[g]. For the assay comparison study, donor sheep (N226) 10 % (w/v) medulla homogenate was diluted using 10% (w/v) uninfected sheep brain homogenate to prepare two dilution series (two-fold dilutions: 5% to 0.078% (w/v); and ten-fold dilutions: 1% to 0.0001% (w/v)). The two-fold dilution series samples were used to compare the sensitivity of the TeSeE sheep/goat ELISA and Western blotting; the ten-fold dilution series was used to compare the sensitivity of real-time quaking-induced conversion (RT-QuIC) assay (details of how the dilution series prepared for RT-QuIC is described in the RT-QuIC method) and Western blot (WB). All samples prepared were passed through 21 gauge and then 25 gauge (G) needles to remove large aggregates. At least quadruplicate homogenates aliquots were prepared from each tissue and stored in a -80^{0}C freezer unless an experiment was planned within 72 hours. Homogenates were vortex-mixed before aliquoting (0.5ml) to maintain a homogeneous condition throughout the experiments. The RT-QuIC seed was solubilised in 500µl of 10% (w/v) medulla homogenate (N226) using 500µl of solubilisation buffer (80% 2X PBS, 170mM NaCl, 0.5% Triton X-100) for 1hr. The homogenate was centrifuged at 2,000G for 2 minutes to collect the supernatant (5%, v/v) as the seed. Serial dilution of the seed used 5 % (v/v) brain homogenate supernatant diluted 1:5 using N2 diluent (1X N-2 supplement (Gibco, 17502048), 0.1% SDS/PBS) into 1% (v/v). Further dilution to make-up the 1:10 dilution series used the N-2 supplement as the diluent (starting with 10^{-2} dilution factor (1%, v/v); diluted to 10^{-10} dilution factor).

Bio-Rad TeSeE Sheep/Goat ELISA kit adapted for quantitative study

The TeSeE sheep/goat purification kit (Bio-Rad, 3551165) and detection kit (Bio-Rad, 3551166) were used to measure PK-resistant PrP^{Sc}. The Omni electronic homogeniser (Camlab) with disposable probes (Omni, 30750) was used to prepare tissue homogenates of various

[g] Fibrous tissue impossible to break down during homogenisation are removed at the end of the homogenisation step.

concentrations (w/v, %). Tissue homogenates prepared in microtubes were thawed for 30 minutes and homogenised by vortexing before use. In order to prepare samples for two-replicate loading, 500µl of proteinase K solution (1: 250 dilutions of TeSeE kit's proteinase K with denaturing solution, R1) was mixed with 500µl of tissue homogenate by inverting ten times, followed by incubation at 37^{0}C in a heating block for 10 minutes. After incubation, 500µl of clarifying solution (R2) was added to the mixture to precipitate PrP^{Sc}. The mixture was mixed homogeneously by inverting ten times as in the step above. Within 30 minutes after mixing with R2 (mixture will turn green caused by an indicator in R2), the mixture was centrifuged in a benchtop microcentrifuge at room temperature (20^{0}C) for seven minutes at 15,000 x g. The supernatant was discarded by inverting microtubes over waste collection tubes. Microtubes were then inverted on an absorbent paper for five minutes before dissolving the pellet in 50µl of resolving buffer (R3). The sample was then heated to denature PrP^{Sc} at 100^{0}C for 5 minutes. The sample was vortexed before proceeding to detection steps or storage for a maximum of 72 hours at -20^{0}C. When defrosting the purified sample from the freezer the sample was thawed to room temperature and vortexed for 1 minute before use. Before starting the detection protocol, all reagents in the TeSeE detection kit were thawed to room temperature for 30 minutes. The purified sample from the TeSeE purification protocol above was diluted with 250µl of sample diluent (R6). Microplate rack and microplate strips[h] enough for each experiment were removed from the closed sachet. TeSeE positive control (R4) was prepared as manufacturer manual before starting the TeSeE detection experiment. A series of truncated ovine ARQ (*PRNP* residues 94 – 233) recombinant PrP (rPrP) standards ranging from 2000 ng/ml to 62.5 ng/ml in two-fold dilution were prepared by dilution in 10mM $NaPO_4$ (pH 5.8). 100µl of standards, positive control, negative control, 10mM $NaPO_4$ (pH 5.8) as the blank, and test samples were loaded in duplicate to R1 strips (inserted into a microplate rack). The double loading process was performed on spleen and PSLN sample[i]. R1 strips were covered with adhesive film before incubating at 37^{0}C for 75 minutes in a shaking incubator. The wash solution was prepared at 1: 10 dilution using the R2 reagent in distilled water. The conjugate solution was then prepared by diluting the peroxidase-labelled anti-PrP monoclonal antibody conjugate (R7) 1:10 in the wash solution. After incubation, the plate was washed using programme "TSE 3" on the plate washer (Bio-Rad, 1575), which consisted of three

[h] R1, each strip contains eight wells coated with anti-PrP monoclonal antibody.

[i] In brief, 100µl of the prepared samples were loaded in each well, the plate was then washed, and a further 100µl of the same samples were added to each well (i.e. equivalent to loading twice the amount of sample per well).

series of washes involving continuous aspiration and dispensing of 800µl wash solution to each ample well. The plate was dried by inverting on absorbent paper before introducing 100µl of the conjugate solution into each well. The plate was covered with adhesive film before incubating at 4^{0}C for 1 hour in the fridge. The colour development solution was prepared by diluting the chromogen (Tetramethylbenzidine solution, R9) 1:11 in peroxidase substrate buffer (R8) and then protected from the light source by covering in aluminium foil). The plate was removed from the fridge or cold room and washed with programme "TSE 5" on the plate washer, which performed five series of washes involving continuous aspiration and dispensing of 800µl of wash solution to each sample well. The plate was dried as per the step above, and 100µl of the colour development solution was distributed into each well. The plate was incubated at room temperature for 30 minutes without covering but protected from light. After incubation, 100µl of stop solution (1N sulphuric acid, R10) was added to each well, causing the colour to change to yellow (if there was a positive signal in the well). The bottom of the plate was wiped dry and the optical density of each well was measured at 450nm and 650nm (bichromatic mode) using POLARstar Omega (BMG Labtech) within 30 minutes of stopping the reaction (always protecting the plate from light). The TeSeE data were analysed by using MARS 3.10 R6 version of data analysis software (BMG Labtech). Blank-corrected optical density (OD) values were calculated by deducting the OD value at 450nm from the OD value at 650nm before correcting all the OD values with the average of the blank value. The TeSeE™ sheep/goat kit specified that a valid test had OD value lower than 0.100 for the negative control and higher than 0.800 for the positive control. A cut-off value for each test was calculated from the mean of negative control plus 0.140 (manufacturer suggested cut-off calculation method).

Quantitative ELISA criteria, quality control and data analysis

The standard curve for rPrP standards was plotted and the curve was fitted using a four parameter logistic (4PL) regression model with the formula below:

$$y = Bottom + \frac{Top - Bottom}{1 + \left(\frac{IP}{x}\right)^{Slope}}$$

Where Top = Highest OD, Bottom = Lowest OD, IP = Inflection point where the slope change course when approaching a horizontal asymptote (top)

Two-fold dilutions of rPrP (truncated ovine ARQ rPrP) standards prepared from 2000ng/ml down to 62.5ng/ml were used to plot a standard curve using 4PL curve fitting. The linear range of the standard curve was used for quantitative study, which referred to the slope between the

top asymptote (top), and the lower cut-off obtain from TeSeE™ sheep/goat kit manufacturer's instruction (OD for negative control + 0.14). Quality control of reproducibility and precision between plates and samples were accessed by calculating inter-assay CV (%) and intra-assay CV (%) respectively. The reproducibility (inter-assay CV, %) was the average of CV (%) from control values, compare between the different experiments. The precision measurement of each experiment was the average CV (intra-assay CV, %) for each sample within the same microplate run. The mean of the controls at the high level (1000ng/ml), intermediate level (500ng/ml) and low level (125ng/ml) were used to calculate the inter-assay coefficient of variation (CV, %). Mean, standard deviation, and CV (%) for each experiment were calculated as CV, % = (Standard deviation / mean) x 100. The functional precision (intra-assay CV, %) for quantitative TeSeE was considered acceptable when (CV) was < 10% and reproducibility (inter-assay CV, %) was < 15% where standard acceptable criteria applied[j]. Any control samples with CV (%) higher than the criteria above were discarded. For data analysis purpose, coefficient of variant (CV,%) > 10% were excluded from the data analysis. Comparison of rPrP equivalent to total protein (PrP^{Sc} level, ng/mg) between genotypes (codon 141) used results calculated from 4PL fitting to get the ratio of total protein (measured with microBCA). Comparison between genotypes was carried out on PSLN, spleen and brain homogenates (w/v, %). Paired student T-test was used to calculate p-values (significant difference between group if $p < 0.05$) using the two-tailed distribution and not assuming equal variances.

Measurement of total protein concentration

Thawed tissue homogenates were diluted in PBS (1:20 or 1:50), depending on the original concentration of the homogenate before measurement using the microplate bicinchoninic acid (microBCA) protein assay kit – reducing agent compatible (Pierce®, 23252). The known standard of bovine serum albumin (BSA) was prepared according to manufacturer's guide using PBS as the sample diluent. 100µl of working reconstitution buffer was diluted (1:1) with distilled water, before being used to dissolve the compatibility reagent. Five microliters of compatibility reagent solution were incubated with nine microliters of each sample and standards (all loaded as duplicate) in 96-well microplates at 37^{0}C for 15 minutes with constant shaking. Working reagent (260µl), consisting of reagent A diluted 50:1 with reagent B (4% cupric sulphate), was added to samples, followed by incubation at 37^{0}C for 30 minutes with continuous shaking. The microplate

[j] Refers to 'Inter- and Intra-Assay Coefficients of Variability' by Salimetrics®

was then cooled at room temperature for five minutes. The absorbance of the microplate was measured at 562 nm on POLARstar Omega (BMG Labtech) plate reader. All OD values were blanked by deducting the average OD of blank wells. The average absorbance (562nm) optical density value was plotted against BSA standards (μg/ml) and a curve was fitted using the second-order polynomial (quadratic) regression model to determine the total protein concentration. Average total protein concentration for each sample was measured using microBCA (μg/ml) and this value was used to calculate the final volume of sample used in each experiment.

Proteinase K digestion and NaPTA precipitation to purify PrP^{Sc}

Ten percent (w/v) brain homogenate (500μl) were thawed and sonicated for ten seconds to break down the cell membrane. Benzonase (Merck, 71205-3) was added to the homogenate to a final volume of 50 units per ml, with 1mM $MgCl_2$ (Sigma, M-8266) to activate benzonase, and incubated at 37^0C for 30 minutes with continuous shaking. An equal volume of 4% (w/v) sodium lauroyl sarcosinate (also known as sarcosyl, Sigma, L-9150) in PBS was added to solubilize and separate the membrane proteins by incubation at 37^0C for 10 minutes with continuous shaking. Proteinase K (Sigma, P-6556) at a final concentration of 50μg/ml was added to the mixture to digest PrP^C, incubating at 37^0C for 75 minutes with constant agitation. 11μl of phenylmethanesulfonyl fluoride (PMSF, Sigma, P-7626) diluted in methanol at 1:100 was added to the mixture to stop the proteinase K activity by vortexing at one minute intervals for five minutes. 96μl of pre-warmed NaPTA solution was added and incubated at 37^0C overnight to precipitate PrP^{Sc}. Samples were centrifuged at 16,000 x g for 30 minutes and the supernatants removed. The pellet was washed with 200μl of 0.1% w/v sarcosyl in PBS and 200μl of 250mM ethylenediaminetetraacetic acid (EDTA, Fisher Scientific, BP2482-1). The pellet was vortexed briefly before repeating the centrifugation step as above to remove supernatant, which contained magnesium chloride precipitation. If the samples contained excess white precipitate, 400μl of 250mM EDTA was used to chelate insoluble magnesium ions by repeating the vortexing and centrifugation steps described as above. 100μl of 0.1% sarcosyl in PBS was used to solubilise the PrP^{Sc} pellet. The pellet was heated at 100^0C for 5 minutes and brief sonication was required to solubilize PrP^{Sc} in sarcosyl solution.

SDS-PAGE and Western blotting

10μl of the sample purified from the experiment above was mixed with 7 μl NuPAGE™ lithium dodecyl sulphate (LDS) sample loading buffer (Invitrogen™, NP0007), and 3 μl NuPAGE™

sample reducing agent (Invitrogen™, NP0009) to maintain a reduced condition during electrophoresis. Samples were run on NuPAGE™ 10% Bis-Tris protein gels (Invitrogen™, NP0315Box) at 160V constant for one hour in reducing conditions by adding 500µl antioxidant to MES SDS running buffer. A combination of protein molecular markers were run in each SDS-PAGE experiment. An Immun–Blot® polyvinylidene difluoride (PVDF) membrane (Bio-Rad, 1620174) was activated in methanol for 30 seconds and incubated with two extra thick blot filter papers (Bio-Rad, 1703968) in transfer buffer containing 50mM Tris-Base (Fisher Scientific, BP152-1), 40mM Glycine (Sigma, 33226) and 20% v/v methanol in distilled water. The gel was transferred onto PVDF membrane and sandwiched with filter paper before transferring the protein from the gel onto PVDF membrane using Trans-Blot® SD semi-dry transfer cell (Bio-Rad) at a constant 20V (112mA) for one hour. The PVDF membrane was blocked in 5% (w/v) skimmed milk powder (Marvel) in PBS containing 0.05% (v/v) Tween-20 (Sigma, P1379) at room temperature for one hour with constant shaking. Blocking buffer was drained before incubating with 0.5µg/ml of the ROS-BC6 anti-PrP antibody prepared in wash buffer (PBS containing 0.05% (v/v) Tween-20) at 4ºC for two hours[k]. The PVDF membrane was washed at least three times for 30 minutes with wash buffer on a rocking platform, followed by an hour of incubation at room temperature with constant shaking with 0.05µg/ml of goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Sigma, A3673) diluted in PBS containing 0.05% (v/v) Tween-20. Washing steps were repeated as above, and the membrane was incubated with 2ml of Clarity™ Western ECL blotting substrate (Bio-Rad, 1705060) for five minutes at room temperature, during which time it was protected from the light source. The membrane was then sandwiched with acetate sheets or cling film before transfer onto an Amersham Hypercassette Autoradiography Cassette (GE Healthcare, RPN11644), and exposed to Amersham Hyperfilm ECL (GE Healthcare, 28906836) for between 30 seconds to 30 minutes, before developing the film using a medical film processor (Konica, SRX-101A). The image of the film was scanned using an HP Scanjet 4850 scanner at 300 dots per inch into a TIFF file using the greyscale setting.

Recombinant PrP protein production

E. coli Rosetta cells (EMD Biosciences) carrying pET41 vector (EMD Biosciences) with hamster-

[k] If time is restricted the immunoblotting step can be done overnight.

sheep chimeric recombinant PrP[l] were grown in Luria Broth[m] at 37°C (225 rpm constant swirling) overnight. Expression of rPrP was performed by adding 10ml of the overnight express autoinduction system (EMD Biosciences) to 400ml of Terrific Broth containing 400µl of chloramphenicol (34mg/ml) and 400µl kanamycin (50mg/ml) and then culturing at 37°C in a shaking incubator. Absorbance (OD at 600nm) was observed between 3 to 6 hours to reach OD_{600nm} between 0.6 and 0.8. Bacteria cells were induced with 400µl of Isopropyl β-D-1-thiogalactopyranoside (IPTG) and then cultured overnight. Cell pellets from cultures were collected by centrifugation at 12,000 rpm for 15 minutes twice. Cell pellets were frozen at -80°C overnight, thawed and then resuspended in 10ml of lysis buffer per gram of cell pellet to isolate the inclusion bodies. Then, they were denatured with 150ml 8 M guanidine-HCl at pH8 per bottle on a rotator for one hour at 4°C. Denatured inclusion bodies were centrifuged at 12,000 rpm for 10 minutes and the supernatant was collected into a 50 ml tube (1:2) with Ni-NTA Superflow resin (Qiagen). The resin and denatured inclusion bodies (rPrP) was mixed on a roller for one hour before loading into a column on an AKTA fast protein liquid chromatography system (GE Healthcare). The denatured rPrP protein was refolded inside the column using a linear gradient from denaturing buffer to renaturing buffer over 2 hours at a flow rate of 1 ml/min. Next, the protein was eluted with a linear gradient from the renaturing buffer to elution buffer at 1 ml/min for 1 hour. The peak of elution of rPrP from the column come around 30 minutes by measuring rPrP at UV 280nm. The protein fractions were collected in different tubes and transferred into snakeskin dialysis tubing (Perbio, 7kMWCO) for dialysis overnight in dialysis buffer. The concentration of rPrP was determined by measuring absorbance (as described below) and the protein was then aliquoted and stored at 0.2–0.4 mg/ml in a -80°C freezer. Before use in the RT-QuIC, rPrP (included truncated ovine ARQ rPrP) was filtered through pre-wet 100kDa Nanosep mirocentrifuge filter (Pall Life Sciences, OD100C34) with centrifugation at 3,000G for 5 minutes. Absorbance (280nm) was measured using a Nanodrop 1000 and the concentration of filtered rPrP was recalculated according to their molecular weight and extinction coefficient, as described on the next page, before adjusting to 0.25mg/ml stocks.

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[l] Syrian hamster *PRNP* gene residues 23 to 137 followed by sheep *PRNP* gene residues 141 to 234 of the R_{154} Q_{171} polymorphism (accession no. AY907689).

[m] With 50µl 34mg/ml chloramphenicol and 50µl 50mg/ml kanamycin per 50ml.

RT-QuIC reaction setting and data analysis

The RT-QuIC reaction buffer was prepared using several types of reagents. Reaction buffers were filtered using a 0.2μm syringe filter and were inverted five times before use in the RT-QuIC. Seed (2μl) and reaction buffer (98μl) were used in each well for RT-QuIC. The POLARstar microplate reader (BMG Labtech) was run on 700rpm double orbital shaking with incubation at 50^{0}C. The program consisted of repeated intervals of shaking for 1 min followed by rest for 1 min, and fluorescence signals were taken every 15min from the bottom of the clear-bottom, black 96-well plate at 450nm excitation and 480nm emission with 2000 gain, 20 flashes. The reaction was run for 90hrs initially, but the interpretation of results will only be allowed between start to 48hrs.

Baselines and threshold determination:

Relative fluorescence unit (RFU) of the samples were corrected on the blank (ThT) before calculated the average over the replicates. Baseline correction took from average over replicates (n = 4) based on the blank corrected result at first cycle. Time to a threshold (C_t) in hours based on mean baseline correction plus 5 SD.

Controls:

Positive controls, negative controls and rPrP were assessed on each plate in at least quadruplicate. All positive control wells (n = 4) required amplifying within 12 hours. None or less than half of negative control and rPrP blank should amplify within 48 hours. Any samples amplified after 48 hours were categorised as spontaneous reactions (spontaneous PrP aggregation).

Detection range and limitation:

Screening of ovine BSE samples (data not shown) showed that ovine BSE samples at 10^{-2} and 10^{-3} did not amplify to maximum RFU. In contrast, any dilutions from 10^{-4} to 10^{-8} were amplified to maximum RFU within 48 hours. The lower detection limit definition was based on amplification efficiency of the sample with at least > 50% of the wells amplified to maximum RFU within 48 hours.

Calculations

rPrP concentration:

UV absorbance of recombinant PrP protein was measured using a NanoDrop1000 spectrophotometer. Sodium phosphate buffer (10mM, pH6.5) was used as the blank before

measurement of 2µl rPrP samples at 280nm and 320nm. Readings were taken twice and the average of the 280nm minus the 320nm values was calculated according to the Beer-Lambert law below:

$A = \varepsilon cl$ rearrange to $c = A/\varepsilon l$

or c (mg/ml) = $\frac{\text{mean } (A_{280nm} - A_{320nm})}{\varepsilon \text{ x (dilution factor) x molecular weight (MW)}}$

Where A is absorbance, ε is the extinction coefficient[n], c is the concentration in moles/L and l is the path length in cm (standard is 1). Hamster-sheep rPrP molecular weight is 23340.6 and extinction coefficient is 62340. Truncated ovine ARQ rPrP MW is 16822.6 and ε are 23380.

Amyloid formation rate:

The rate at which amyloid formation reached the designated threshold, termed C_t values (*135*), was obtained by determining the time in an hour (h) for the average positive aggregation formed over the threshold in the assay. The amyloid formation rate is calculated from inverted time to reach threshold (one per time to the threshold, 1/h). At least quadruplicate replicates of each serial dilution between 10^{-4} and 10^{-10} were used to determine the linear range of the dilution series. The linear range of each sample was fitted to the logarithmic equation $y = m \log x + b$ to calculate the rate of amyloid formation across end-point dilution series.

Detection limit:

ELISA

Brain weight was calculated to determine the volume used in each microplate well (100µl / well). 10% (w/v) = 50mg brain (in 500µl homogenate) resuspended into 300µl (enough for two replicates).

Brain loaded for each well = 50mg / 3, yielded 16.67mg.

Further calculated for 0.1563% (w/v) detection limit of brain = 781.25µg / 3, yield 260.4µg.

WB

As previously, the brain weight was calculated to determine the protein loading volume for WB (10µl / well).

10% (w/v) = 50mg brain (in 500µl homogenate) resuspended into 100µl.

[n] Obtained from the ExPAXy ProtParam tool to calculate ε, assuming all Cys residues are reduced.

Brain loaded for each well = 50mg / 10, yielded 5mg.

Further calculated for 0.01% (w/v) detection limit of brain = 50μg / 10, yielded 5μg.

RT-QuIC

Brain weight was calculated to determine the protein loading volume in RT-QuIC (2μl / well).

5% (v/v) = 50mg brain (in 1ml solubilised homogenate), taken 10μl to prepare 1% (v/v) sample.

1% (v/v) = 50mg / 100, yielded 0.5mg (in 50μl diluted homogenate).

Brain loaded for each well = 0.5mg / 25, yielded 0.02mg.

Further calculated for 10^{-8} dilution factor detection limit of brain = 500 pictograms / 25, yielded 20pg.

Results

1. Adaptation and optimisation of the ELISA kit for PrP^{Sc} measurement.

Although Western blotting (WB) and immunohistochemistry (IHC) are the conventional methods used for confirmatory testing of TSE diseases in sheep, a rapid screening method such as ELISA is a more efficient assay to detect disease-related PrP^{Sc} with high sensitivity and specificity. ELISA tests such as the IDEXX HerdChek BSE-Scrapie Antigen Test kit and the TeSeE sheep/goat kit are European Commission (EC)-approved rapid tests for the detection of PrP^{Sc} in post-mortem ruminant brain and lymphoid tissues according to Commission Regulation (EC) No 253/2006 and are commonly used in research for TSEs diagnostics (*99, 136-138*). Therefore, the TeSeE sheep/goat kit was chosen for adaptation into a quantitative assay.

1.1. Optimisation of the ovine (sheep) rPrP as the calibrator (standard).

The currently available commercial TeSeE sheep/goat ELISA kit is a diagnostic assay that is not designed for quantification purposes since it requires a proper standard calibrator (rPrP) for use in a quantitative study. Truncated ovine (*PRNP* amino acids 94 - 233) $A_{136}R_{154}Q_{171}$ rPrP (refers as rPrP unless specified)[o] was selected as the standard calibrator among the rPrPs mainly because the protein sequence is equivalent to the misfolded PrP^{Sc}. Optimisation experiments were carried out to determine the rPrP (ng/ml) detectable range in the ELISA kit. In this case, rPrP concentrations between 10ng/ml and 2000 ng/ml were tested (data not shown). The rPrP standard curve was plotted using a four-parameter logistic (4PL) curve fitting (sigmoidal curve) and the threshold for the R-square was set above 0.99 as the acceptance criterion. The standard curve (**Figure 4**) was implemented for the whole study.

The relative amount of PrP^{Sc} was estimated using the equivalent amount of rPrP per total protein of the sample in the quantitative ELISA analysis. The samples were loaded in duplicate and calculated using a standard curve plotted with at least five standards ranging from 2000ng/ml to 62.5ng/ml (two-fold dilutions). A 4PL sigmoidal model was used for this study due to the notable S-shaped curve shown by rPrP standards (*139, 140*). In order to obtain comparable data, only the linear range of the standard curve (highlighted in **Figure 4**) between the cut-off value and horizontal asymptote (top) was used. The outcome was shown as the relative amount of PrP^{Sc}

[o] The rPrP was obtained from a stock prepared by former group member (Dr R. Blanco) using the same method as per hamster-sheep chimeric rPrP.

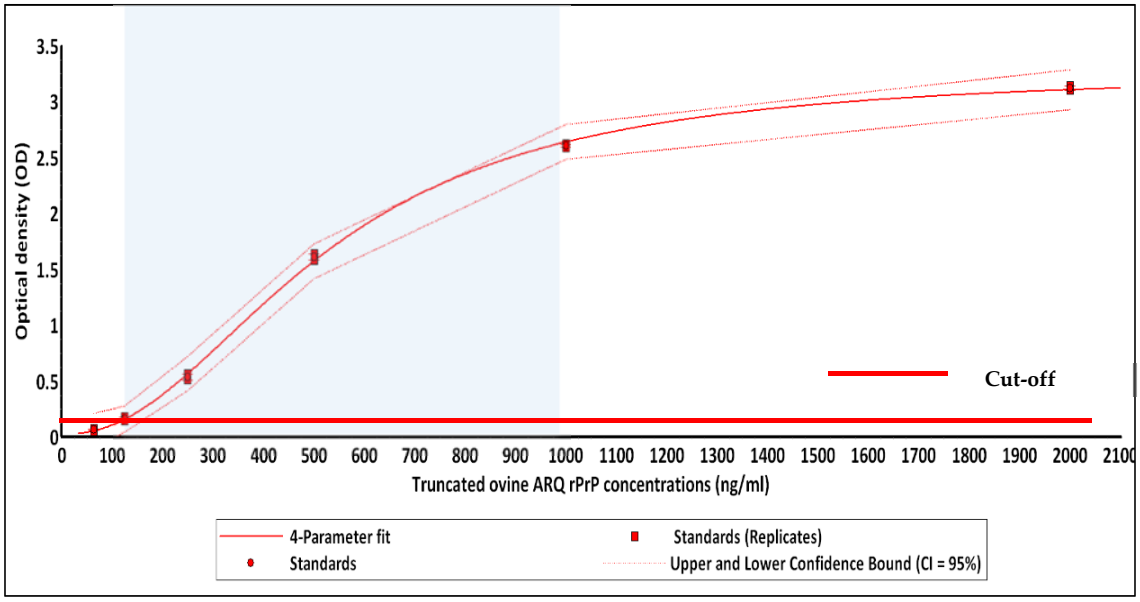

Figure 4: Optical density (OD) against rPrP concentration (ng/ml) standard curve.
The figure represents the ovine truncated ARQ rPrP standards that were prepared as two-fold dilutions from 2000ng/ml to 62.5ng/ml. The highlighted region is the linear range of OD values used in data analysis. The results out of the linear range were excluded from data analysis. The red line indicates the cut-off value according to TeSeE™ sheep/goat kit manufacturer's instruction (OD for negative control + 0.14).

(refers as PrP^{Sc} level) or rPrP-to-TP (ng/mg) in BSE-infected samples. Any samples with optical density (OD) values outside the linear range were excluded from the data analysis.

1.2. Optimal concentration (%, w/v) of the brain and lymphoid tissue (spleen) samples used in quantitative ELISA.

Prior to the experiments, the concentrations of the BSE-infected brain and lymphoid homogenates were assessed by dilution in non-BSE homogenates (20%, w/v). BSE-infected brain samples were prepared to give final concentrations of 20%, 15%, 10%, 7.5%, 5%, 2.5% and 1.25% (w/v). The results (**Figure 5**) indicated that BSE-infected brain homogenates at 5%, 7.5%, 10% and 15% (w/v) showed OD values within the linear range while brain at 20% (w/v) concentration reached the horizontal asymptote (top). This experiment demonstrated the optimal concentrations of brain homogenates for quantification were around 5% to 15% (w/v). To allow for the variation in PrP^{Sc} deposition among different sheep, a 10% (w/v) was initially used in the experiments.

For the experiments to test BSE-infected spleen homogenates, samples prepared at 5%, 7.5%, 10%, 15% and 20% (w/v) were analysed (**Figure 6**) as per the previous experiment. The findings indicated that the levels of PrP^{Sc} in BSE-infected spleens were, as expected, much lower than in

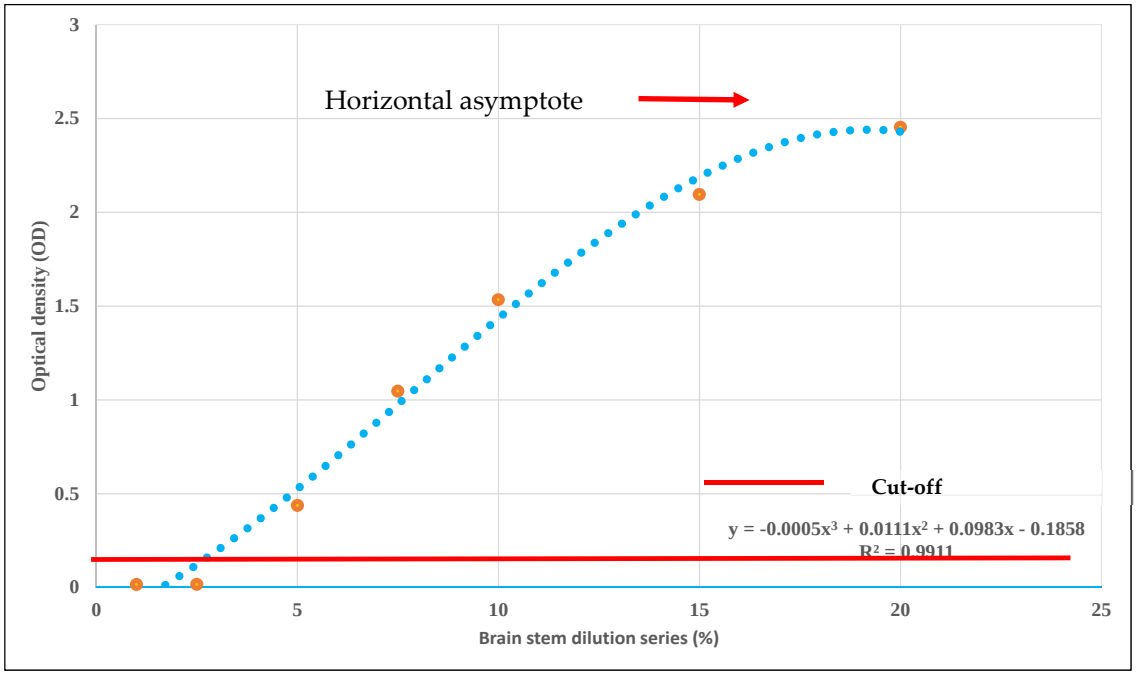

Figure 5: Dilution series of BSE-infected brain homogenates (w/v, %).
A BSE-infected brain dilution series ranging from 1.25% to 20% (w/v) was evaluated to determine the optimal concentrations for detection of PrP^{Sc}. The red line indicates the cut-off value according to the TeSeE™ sheep/goat kit manufacturer instructions (OD for negative control + 0.14). The red arrow shows the horizontal asymptote (top).

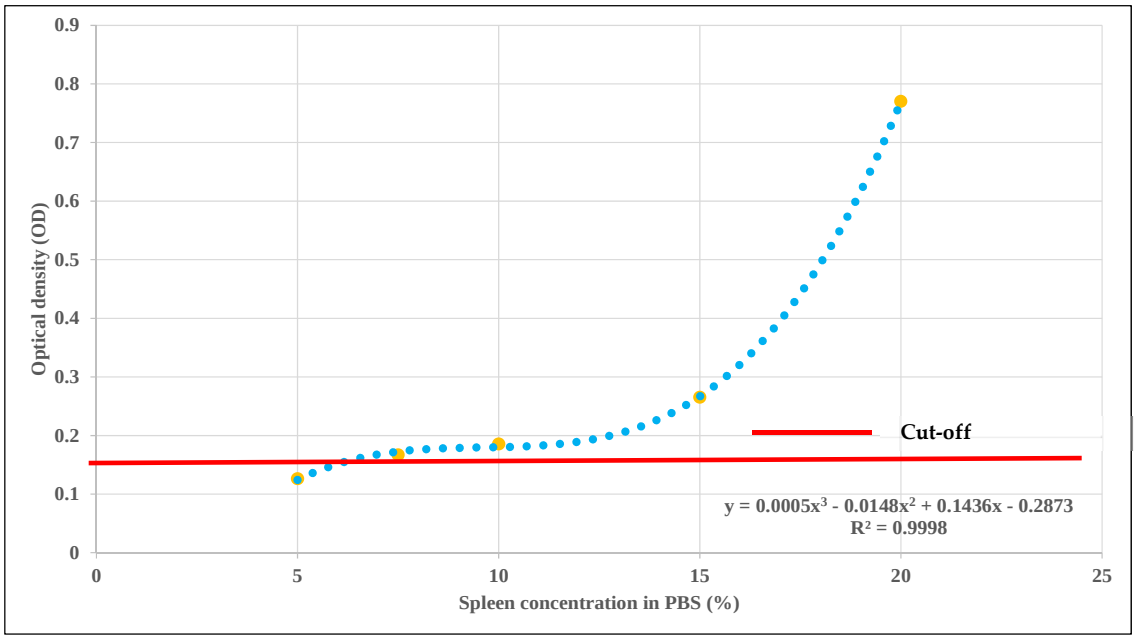

Figure 6: Dilution series of BSE-infected spleen homogenate (w/v, %).
A dilution series of BSE-infected spleen ranging from 5% to 20% was evaluated for detection of PrP^{Sc}. The red line indicates the cut-off value according to TeSeE™ sheep/goat kit manufacturer instructions (OD for negative control + 0.14).

the brains; in fact, the only concentration of spleen homogenate that fell within the linear range of the standard curve was 20% (w/v). Spleen samples at ⩽ 15% (w/v) were close to the cut-off value, meaning that spleens prepared at this concentration range were not suitable for the study. A follow-up experiment showed that double loading of 20% (w/v) spleen homogenate resulted in increased OD signals from the TeSeE sheep/goat kit (**Appendix 4**), although one of the spleen samples was still under the cut-off despite such an increase. Loading spleen samples at ≥ 20% (w/v) is a potential alternative option but the high viscosity caused by such concentrations would present some technical issues.

1.3. Quality control (QC) assessment of quantitative ELISA.

Quality control criteria for assessing reproducibility and precision of an ELISA assay were measured by calculating inter-assay and intra-assay coefficients of variation (CV, %) (*141*). The CV (%) was calculated using the formula below:

$$CV\ (\%) = \frac{SD}{Mean} \times 100$$

The higher the CV (%) value the greater the error in the assay. Some clinically used immunoassays have been validated within a 20% criterion but there is no general agreement regarding the validity of these criteria (*142, 143*). The intra-assay CV (%) was measured simultaneously in all tests for precision using a standard assay criterion. The maximum acceptable CV (%) for intra-assay variability (precision) was set at 10% (*139*) and inter-assay variability (reproducibility) was set at 15% (*141*). Standard calibrators that had technical replicates with a CV (%) higher than 10% were discarded from the curve fitting. Similarly, test samples and positive controls within the ELISA microplate showing a CV (%) more than 10% were excluded from the data analysis (*144*). As an example, the result of intra-assay CV (%) for experiment G (**Table 1**) showed OD values for two replicates of 5% (w/v) BSE-infected brain homogenates.

For the intra-assay variability, the average CV (%) was calculated from the mean and standard deviation (SD) of the OD values. An average result of 2.75% (≤ 10% criteria) exhibited the average variation among seven brain samples (5%, w/v) tested in experiment G. Additionally, the intra-assay CV for other experiments was between 2.75% and 8.58% (**Appendix 2**). The reproducibility of an assay (inter-assay CV, %) was determined by measuring the CV (%) for calibrator levels (determined as the control high, intermediate and low) in **Table 2** to three

Sample	Result 1	Result 2	Mean	SD	CV (%)
1	2.53	2.74	2.63	0.15	5.72
2	2.35	2.52	2.43	0.13	5.20
3	2.65	2.62	2.63	0.02	0.75
4	1.83	2.00	1.91	0.12	6.33
5	2.29	2.27	2.28	0.01	0.50
6	3.44	3.41	3.43	0.02	0.70
7	2.40	2.40	2.40	0.00	0.03
			Intra-assay CV (average CV, %)	(N = 7)	2.75

Table 1: Intra-assay coefficient of variation (CV, %) for experiment G.

Experiment	Control high (OD)			Control intermediate (OD)			Control low (OD)		
	Result 1	Result 2	mean	Result 1	Result 2	mean	Result 1	Result 2	mean
A	3.04	2.84	2.94	1.86	1.93	1.89	0.19	0.22	0.21
B	3.27	3.18	3.22	2.26	2.31	2.28	0.21	0.20	0.21
C	2.56	2.57	2.57	2.08	1.45	1.77	0.28	0.18	0.23
D	3.06	3.06	3.06	2.13	2.08	2.11	0.36	0.32	0.34
E	2.67	2.74	2.71	1.98	1.92	1.95	0.33	0.23	0.28
Mean of means			2.90			2.00			0.25
SD of means			0.26			0.20			0.06
CV (%)			9.11			10.03			22.14
Inter-assay CV (%)			13.76						

Table 2: Inter-assay coefficient of variation (%) of five independent experiments.

different calibrator levels in order to determine the variation in optical densities across the five independent experiments. The standard calibrator at 125ng/ml (control low), 500ng/ml (control intermediate) and 1000ng/ml (control high) used in each experiment are listed; the means of both OD and SD were used to calculate the CV (%) and the average CV for each concentration were determined in order to give the inter-assay CV (%). The inter-assay CV (%) for the experiments (n = 5) was 13.76% (≤ 15% standard acceptable criteria), which does reflect the reproducibility of these experiments. Even though the inter-assay CV was within standard acceptable criteria, the fact that the mean CV% was over 10% showed that rPrP standards display some variation between experiments. Therefore, this have caused an issue especially for tissues with higher PrP levels such as the brain.

2. Relative amount of PrP^{Sc} in the brain and lymphoid tissues (PSLN and spleen).

2.1 Assessment of the differences in PrP^{Sc} levels between PRNP codon 141 genotypes.

As mentioned in the project background, PrP^{Sc} distribution in various types of lymphoid tissue (tonsil, spleen, prescapular lymph nodes, mesenteric lymph node and distal ileal Peyer's patches) had been determined previously in a semi-quantitative manner by immunohistochemistry. The IHC findings suggested that the relative PrP^{Sc} levels observed in the lymphoid tissuestissues (as assessed by the number of tissues showing positive staining by IHC) were influenced by the *PRNP* codon 141 genotype of the host (sheep). The ELISA method was used to assess the differences in PrP^{Sc} deposition in relation to the codon 141 genotypes. Relative PrP^{Sc} level was estimated according to the standard calibrator (rPrP) divided by the total protein amount, which was measured using microBCA (data not shown) for each sample. The PrP^{Sc} level results from different tissues were used to compare among the codon 141 genotypes. The results within the linear range of the standard curve (**Figure 4**) were used in data analysis; and only samples with coefficient of variation (CV,%) < 10% were used for the data analysis.

2.2 PrP^{Sc} levels in BSE-infected brain and peripheral tissues (spleen and PSLN) according to *PRNP* codon 141 genotypes.

BSE-infected brain homogenates from 16 sheep were prepared at 10% and 5% (w/v) concentrations for quantitative ELISA tests. The homogenate samples for genotypes LL_{141} (n = 5), FF_{141} (n = 6) and LF_{141} (n = 5) were loaded in duplicate into microplate wells. The estimated levels of PrP^{Sc} in the same animal for experiments A, B and C (**Table 3**) were inconsistent (see **Appendix 3** for more details), which might have been caused by the loss of PrP^{Sc} protein during preparation of purified PrP^{Sc}. PrP proteins are notorious for their adhesiveness properties which prove hard to remove during the conventional decontamination process(*145*). Therefore, loss of PrP during multiple extraction steps can be a major issue.

The relative PrP^{Sc} in the lymphoid tissues were compared to the brain and showed that the PrP^{C} protein expression in tissues type actually affects the levels of PrP^{Sc}. Researchers have previously shown that the PrP^{Sc} level in peripheral lymphoid tissues such as spleen and tonsil can be 2 to 3 logs lower than in the brain (*82*, *87*, *133*). In experiments D and E (**Table 3**), BSE-infected samples of PSLN and spleen from 16 sheep with genotype LL_{141} (n = 6), FF_{141} (n = 5) and LF_{141} (n = 5) were tested. The LF_{141} sheep lymphoid tissues consistently gave signals below the cut-off value for the

Tissues (%,w/v)	Experiment	Genotypes (numbers of sheep within linear range, N)	PrP^{Sc} level (ng rPrP equivalent/mg total protein (mean ± SD)	Paired T-test (p value)
Brain (10%)	**A**	LL_{141} (4/5)	22.9 ± 8.8	0.28 (LL/FF)
		FF_{141} (4/6)	36.8 ± 20.3	0.37 (FF/LF)
		LF_{141} (3/5)	24.9 ± 11.6	0.82 (LL/LF)
Brain (10)	**B**	LL_{141} (5/5)	85.2 ± 32.8	0.94 (LL/FF)
		FF_{141} (6/6)	83.4 ± 50.5	0.74 (FF/LF)
		LF_{141} (5/5)	74.9 ± 29.6	0.62 (LL/LF)
Brain (5)	**B**	LL_{141} (4/5)	65.6 ± 10.2	0.34 (LL/FF)
		FF_{141} (4/6)	107.7 ± 74.9	0.76 (FF/LF)
		LF_{141} (4/5)	93.8 ± 43.4	0.29 (LL/LF)
Brain (5)	**C**	LL_{141} (4/5)	41.7 ± 11.8	0.49 (LL/FF)
		FF_{141} (6/6)	52.3 ± 32.6	n.a
		LF_{141} (2/5)	61.1, 23.1	n.a
Spleen (20 x 2)	**D**	LL_{141} (4/6)	3.0 ± 0.3	n.a
		FF_{141} (2/5)	3.8, 3.3	n.a
		LF_{141} (0/5)	< 2.4	n.a
PSLN (20 x 2)	**D**	LL_{141} (5/6)	11.5 ± 5.3	0.30 (LL/FF)
		FF_{141} (5/5)	8.4 ± 2.7	n.a
		LF_{141} (0/5)	< 2.4	n.a
PSLN (20)	**E**	LL_{141} (6/6)	12.7 ± 7.2	0.12 (LL/FF)
		FF_{141} (4/5)	6.7 ± 2.9	n.a
		LF_{141} (0/5)	< 2.4	n.a

Table 3: Comparison of PrP^{Sc} levels in BSE-infected tissues of the different *PRNP* 141 genotypes.
N indicates the numbers of animals that qualified for data analysis after QC. Paired student T-tests using two-tailed distribution and not assuming equal variances were carried out to compare each sample group on codon 141 genotypes. Not applicable (n.a.).

ELISA, and therefore their relative PrP^{Sc} levels could not be measured (estimated from **Figure 8** in section 4 as 2.4 ng/mg). The tendency for PSLN tissues to have higher relative PrP^{Sc} levels than spleen may again reflect the variation in PrP expression among different tissues. The means, SDs and paired student T-test results for brain, spleen and PSLN are summarised in **Table 3.** PSLN samples loaded at 20% (w/v) gave similar results for the homozygote genotypes – relative PrP^{Sc} levels were 11.50 ± 5.35 (mean ± SD; double loading) and 12.69 ± 7.24 (single loading) for LL_{141} and 8.44 ± 2.67 (double loading) and 6.77 ± 2.93 (single loading) for FF_{141}. For double loading of the spleen, homozygote L_{141} and F_{141} relative PrP^{Sc} levels were at low levels of 3.09 ± 0.34 and 3.58, respectively. There was no significant difference between the results obtained by single loading or double loading of 20% (w/v) PSLN samples for LL_{141} and FF_{141} genotypes, confirming that

double loading does not improve the sensitivity of the assay. PSLN samples from animals of the codon 141 homozygote seemed to contain similar PrP^{Sc} levels and the same trend was observed for the spleen samples. Importantly, PrP^{Sc} levels in heterozygote LF_{141} samples of both PSLN and spleen were below the cut-off (low) value.

Paired student T-tests using two-tailed distribution with unequal variance were used to compare the relative PrP^{Sc} levels for brain and PSLN with the condition that each sample group had to have at least three valid results. No genotype comparisons showed statistically significant differences for the brain tissues, probably because some of the genotype groups had high SD values caused by variation between the repeat of same sample. The p-values for single loading and double loading of PSLN showed that relative PrP^{Sc} levels did not differ significantly between the homozygote 141 genotypes ($p > 0.05$), as was the case for the brain tissues. Collectively, the preliminary observations showed that relative PrP^{Sc} levels (ng/mg) obtained from peripheral tissues such as PSLN and spleen were consistently under the TeSeE sheep/goat kit cut-off value for the heterozygote LF_{141} animals. Future work could involve testing all lymphoid tissues, including mesenteric LN, tonsil and distal ileal Peyer's patches. Because both spleen and PSLN from heterozygote LF_{141} animals displayed relative PrP^{Sc} levels under 2.4ng/mg, a more sensitive assay for quantification was required. Therefore, Western blot was chosen as a potential alternative technique to quantify relative PrP^{Sc} level.

3. Comparison of the sensitivity of ELISA and Western blot.

Although ELISA is commonly used for the quantitative studies, assay methods such as the Western blot may serve as an alternative with the aim of quantifying PrP^{Sc} level. In order to look into the difference in sensitivity between ELISA and WB, BSE-infected brain homogenates were prepared in two separate dilution series (two- and ten-fold dilutions) using uninfected brain homogenate (20%, w/v). The two-fold dilution series (10% to 0.078%) was used to compare the detection limits (minimum brain material needed) of the Western blot and the ELISA. The WB results (**Figure 7a**) showed a weak PK-resistant PrP^{Sc} band in lane 7, which became a distinct band after longer exposure (data not shown), indicating that the detection limit for WB was 0.01% (w/v). In parallel, the experiments using a two-fold dilution series (**Figure 7b**) of the BSE-infected brain led to gradually decreasing levels of PrP^{Sc} bands similar to the ten-fold dilution series. Therefore, two different serial dilutions (two- and ten-fold) of the BSE-infected brain

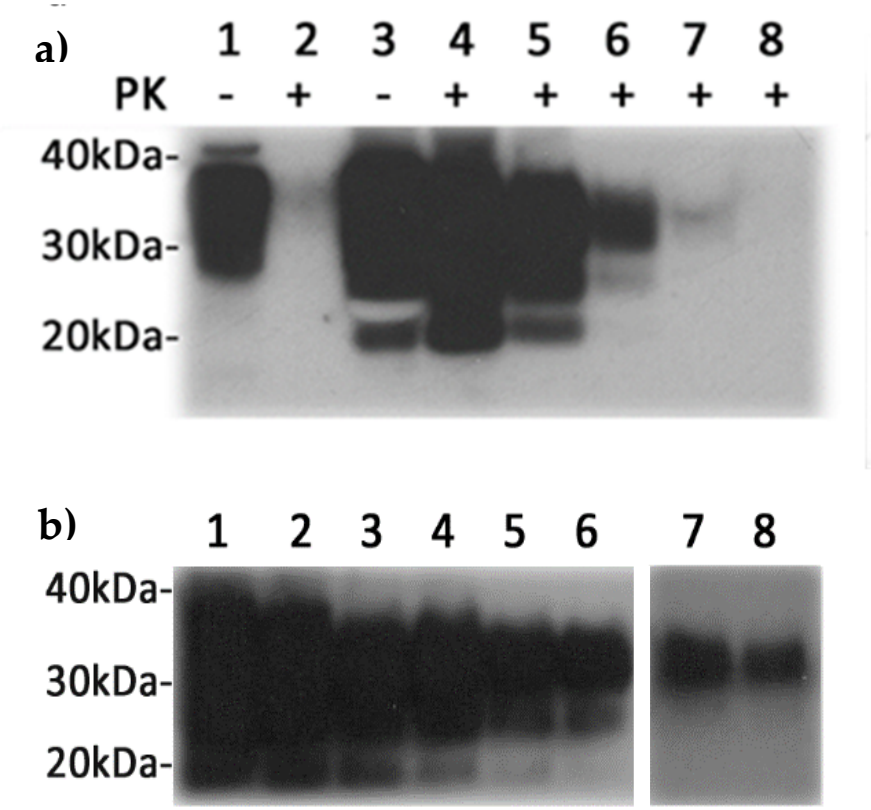

Figure 7: PrP^{Sc} expression of proteinase K (PK) treated and non-PK treated (ten-fold only) brain homogenate in Western blot.
PrP^{Sc} expression level in brain homogenate samples was detected by ROS-BC6 anti-PrP antibody at a concentration of 0.5µg/ml. Lanes 1 and 2 in **Figure 7a** are 10% (w/v) uninfected brain homogenate; lane 3 was 10% BSE-infected brain; lanes 4 to 8 were ten-fold dilution series of BSE-infected brain; "PK (-)" corresponds to non-PK treated samples and "PK (+)" to PK-treated samples; lanes 4 to 8 were 10%, 1%, 0.1%, 0.01% and 0.001% (w/v) respectively. **Figure 7b** shows PK-treated BSE-infected brain homogenates, with lanes 1 to 8 showing a two-fold dilution series at a concentration of 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156% and 0.078% (w/v) respectively. The image of lanes 7 and 8 was the continuation of the dilution series on a separate blot.

demonstrated that the lower limit of brain material required for PrP^{Sc} detection in WB was approximately 5µg (10µl per well), with the detectable range between 5µg and 5,000µg. When using rPrP as the calibrator, the detection limit of ELISA (adapted from TeSeE™ sheep/goat kit) was shown (**Figure 8**) at 0.16% (w/v) or approximately 260.4µg of the brain (100µl per well). The range of relative BSE-infected brain material needed for ELISA (TeSeE™ sheep/goat kit) was between 260.4µg and 17,000µg.

4. Optimisation of RT-QuIC assay for detection of PrP^{Sc} in BSE-infected sheep brain.

RT-QuIC was required to optimise on ovine BSE detection before it could be used for quantification of PrP^{Sc} in the blood components for future work. An optimisation plan would be to determine the most suitable rPrP as the substrate to seed brain PrP^{Sc} amplification before one could optimise the sensitivity for lymphoid tissues and even blood components, TSEs strain,

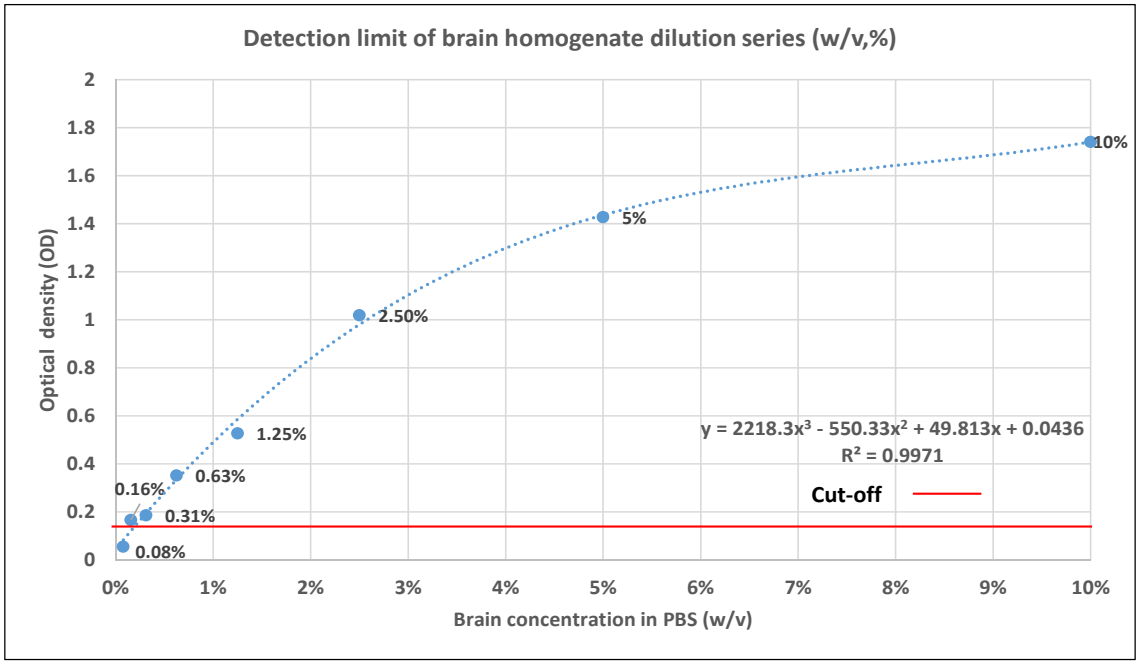

Figure 8: Detection limit of BSE-infected brain prepared in two-fold dilution series (w/v, %). Values from eight concentrations of the BSE-infected brain were plotted to the best-fitting curve with $R^2 = 0.99$. The lowest detectable concentration was 0.16% (w/v). The red line indicates the cut-off value suggested by the manufacturer's instructions (OD for negative control + 0.14).

optimal temperature and seeding period.

4.1. Effect of different rPrP (substrate) and reaction temperatures in the RT-QuIC.

Optimisation of the real time quaking induced conversion (RT-QuIC) was carried out in order to determine the required substrate (rPrP) for conversion into the amyloid seed (PrP^{Sc}) as well as the optimal temperature for the amyloid seeding reaction. To assess the PrP^{Sc} seeding activity, seed and/or converted seed (from the substrate, rPrP) was detected by positive fluorescence or RFU (signal giving out by the binding of thioflavin T to the amyloid structure) during the amplification process in the RT-QuIC. The maximum signal exhibited within the set cut-off time of 48 hours was considered as a positive amyloid seeding reaction (refer to RT-QuIC data analysis). Otherwise, any amplification of amyloid seeding after 48 hours or not reaching maximum signal was considered as a spontaneous reaction and/or a negative result (the cut-off time may differ between laboratories though generally falls between 45 and 55 hours). The hamster-sheep chimeric recombinant PrP (HSrPrP) was produced and tested previously by the Caughey group to detect PrP^{Sc} in vCJD (*129*) and classical scrapie (*125*) samples. This suggested that HSrPrP is a potential substrate for amplification of PrP^{Sc} in ovine BSE samples. To determine the sensitivity

and specificity of HSrPrP in RT-QuIC, natural scrapie (data not shown), mouse and/or hamster-adapted scrapie were used during the optimisation studies. Scrapie strain 263K was selected as the positive control because it is the most sensitive seed for the RT-QuIC reaction and can be amplified with various types of substrate (rPrP) within 12 hours (*131, 132*). The RT-QuIC results showed that HSrPrP successfully amplified the 263K (positive control) samples but not the ovine BSE brain samples (**Figure 9**). Next, the different types and/or batches of rPrP were tested, which included truncated ovine $A_{136}R_{154}Q_{171}$ rPrP. The findings indicated that the truncated ovine $A_{136}R_{154}Q_{171}$ rPrP (**Figure 10 and 11**) served as a more favourable substrate compared to HSrPrP.

Since evidence has reported that temperature might be one of the factors that can influence the RT-QuIC reaction (*131*), subsequent tests of reaction temperatures at 42^{0}C and 50^{0}C were carried out (**Figure 10a and 10b**). An independent positive control – 79 A (mouse-adapted scrapie) was used with rPrP (truncated ARQ rPrP) substrate and the results showed that the PrP^{Sc} amplification time was also within 12 hours similar to 263K. In **Figures 9, 10 and 11**, the same colour codes were used for indicating 263K, 79A, uninfected, blank (rPrP only) and ovine BSE-infected brain dilution series (10^{-4} to 10^{-6}) throughout the experiments. The substrate reacted with 263K to form amyloid seed in RT-QuIC (increasing of ThT fluorescence signal) in a shorter duration time of 3 hours at 50^{o}C (**Figure 10b**) and 4 hours at 42^{0}C (**Figure 10a**). In parallel, 79A showed amplification at approximately 5 hours for both temperatures. The observations also demonstrated that the substrate was amplified to the amyloid form more consistently at 50^{0}C compared to 42^{o}C in the case of ovine BSE. This was shown by some of the unstable and inconsistent seeding reaction after 24 hours for some dilutions (**Figure 10a**, 10^{-6}) at the lower temperature (42^{o}C). At the same time, when the RT-QuIC reaction was tested at 50^{0}C, none of the negative control reached the maximum ThT fluorescence signal (within 48 hours). Overall, the RT-QuIC results showed that the amyloid seeding reaction was more stable at higher temperatures (50^{0}C).

4.2 Analysis of PrP^{Sc} amyloid formation rate.

Amyloid seeding reaction was evaluated using an endpoint dilution of ovine BSE-infected brain homogenates (10^{-4} to 10^{-10}) (**Figure 11**). The infected sample dilutions between 10^{-1} and 10^{-3} generally exhibited no or low amyloid seeding in the assay due to their high concentration (*135*). BSE-infected samples were prepared at ten-fold serial dilutions to define the limit of the RT-QuIC

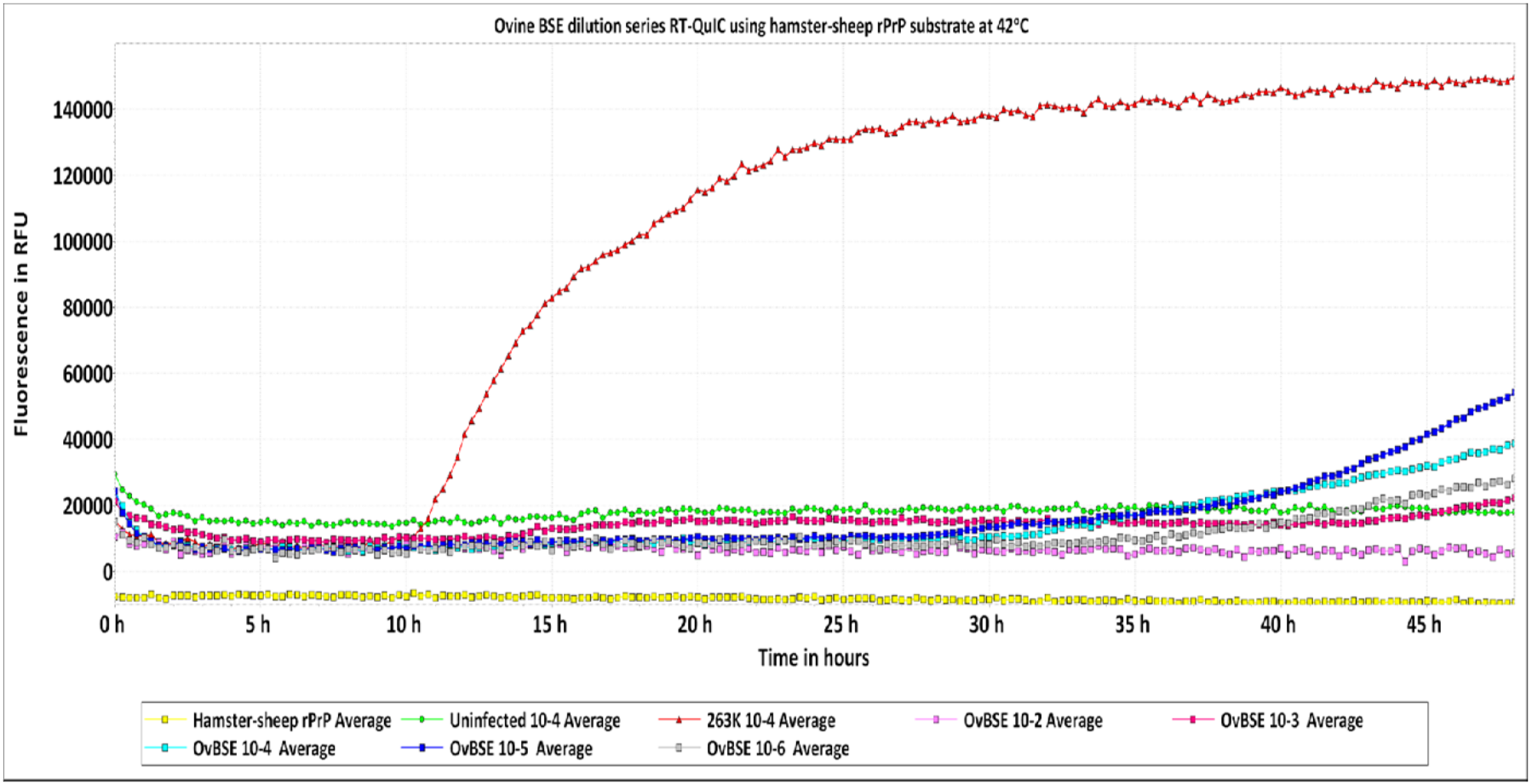

Figure 9: RT-QuIC reaction data for ovine BSE-infected brain dilution series amplification using hamster-sheep rPrP at 42°C.
Figure shows the fluorescence signals from PrP^{Sc} amyloid seeding reactions of ovine BSE brain homogenate for 48 hours. 263K (10^{-4}) acts as positive control and uninfected sheep brain (10^{-4}) was used as negative control. The colour-coded lines shown in the figure represent the averages of the quadruplicate samples.

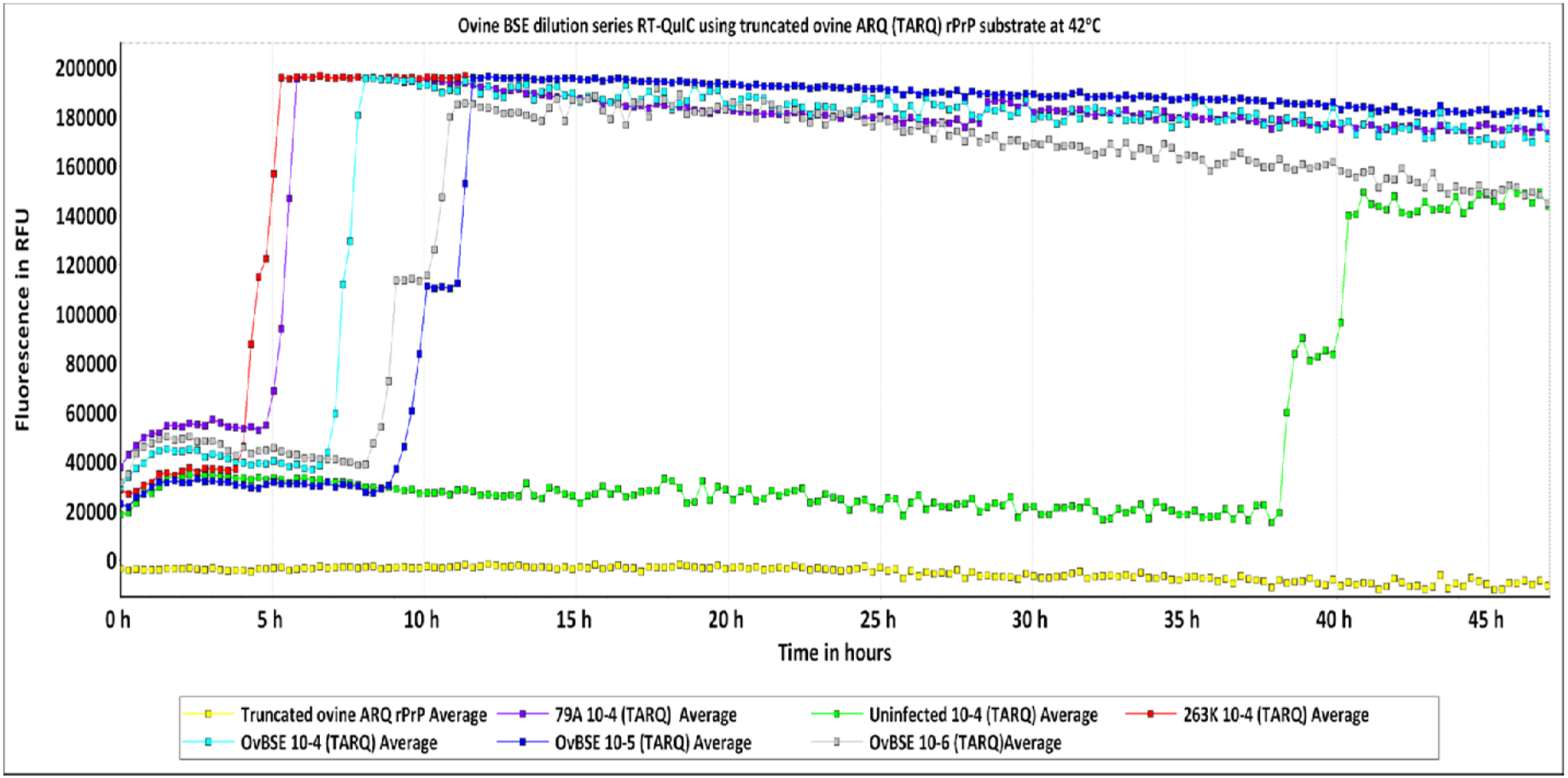

Figure 10a: RT-QuIC for ovine BSE-infected brain dilution series amplification using truncated ovine ARQ rPrP at 42°C.
Figure shows the fluorescence signal from PrP^{Sc} amyloid seeding reactions of ovine BSE brain homogenate for 48 hours. Both 263K (10^{-4}) and 79A (10^{-4}) act as positive control and uninfected sheep brain (10^{-4}) was used as negative control. The colour-coded lines shown in the figure represent the averages of the duplicate samples.

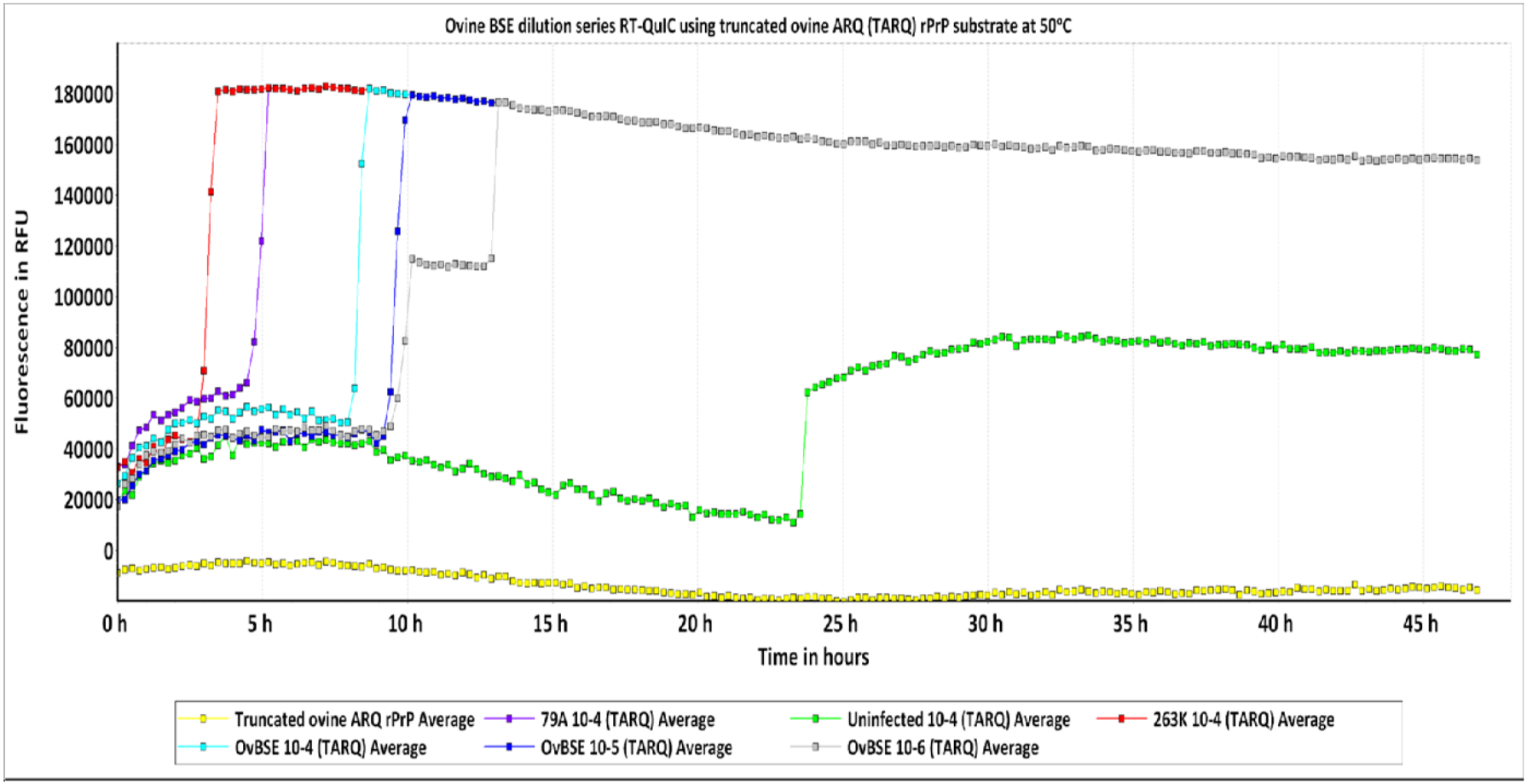

Figure 10b: RT-QuIC for ovine BSE-infected brain dilution series amplification using truncated ovine ARQ rPrP 50°C.
Figure shows the fluorescence signals from PrP^{Sc} amyloid seeding reactions of ovine BSE brain homogenate for 48 hours. Both 263K (10^{-4}) and 79A (10^{-4}) act as positive control and uninfected sheep brain (10^{-4}) was used as negative control. The colour-coded lines shown in the figure represent the averages of the duplicate samples.

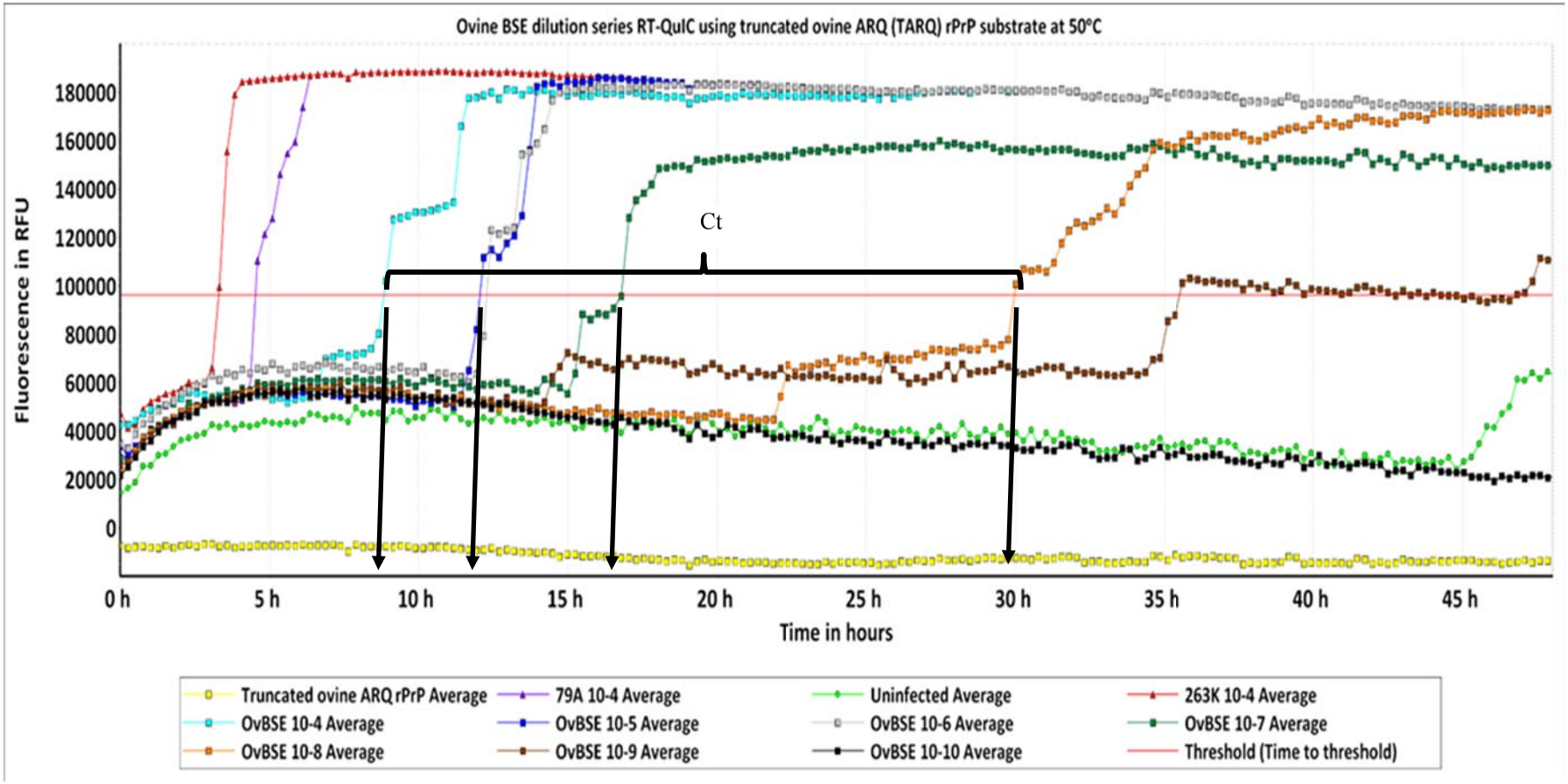

Figure 11: RT-QuIC of ovine BSE brain dilution series amplification using truncated ovine ARQ rPrP at 50°C.
Figure shows the fluorescence signals at 48 hours from the PrP^{Sc} amyloid seeding reactions for ovine BSE brain homogenates at the 42°C reaction temperature. 263K and 79A both at 10^{-4} were used as positive controls and uninfected sheep brain (10^{-4}) was used in the RT-QuIC reaction. The colour coded lines shown in the figure represent the averages of the quadruplicate wells for each sample. The red line was the threshold level calculated as the mean baseline signal plus 5 SD. The rate of amyloid formation was determined as the inverse of the time (1/h) for an aggregation to reach threshold level.

for detecting a positive reaction. Although blank and uninfected controls occasionally exhibited spontaneous aggregation, none of them reached the maximum fluorescence signal by the 48 hours cut-off time. Amyloid seeding reactions of ovine BSE-infected brain homogenates (seeds) were observed with amplifying amyloid sequentially from 10^{-4} to 10^{-8}. The seed at 10^{-9} dilution only showed positive amyloid seeding in two out of four (quadruplicate) wells within 48 hours and none of the signals reached maximum fluorescence within 48 hours. Thus, it can be suggested that the sample at this dilution (10^{-9}) was a negative finding. The RT-QuIC detection limit for the ovine BSE-infected brain to form amyloid seed was determined to be the 10^{-8} dilution.

The amyloid seeding rate model was employed to quantitatively estimate the PrP^{Sc} level and/or infectivity titre of TSE-infected samples (*135*). The seeding activity was positively correlated to the lag phase (time in hours) before reaching fluorescence threshold level. The rate of amyloid seeding was determined by using the inverse time (1/h) of an aggregation to reach the threshold level (C_t), calculated as the mean baseline fluorescence signal plus 5 SD (*135*). This hypothesised that when the amyloid seeding forms below the detectable level, there was no distinct signal unless amyloid seeding started to form aggregates (prion hypothesis) over the target baseline (background) and threshold level, which is similar to the lag phase observed in RT-PCR. The amyloid seeding model has been shown with linear range when the amyloid seeding rate or inverse time (1/h) was plotted at logistic regression against serial dilutions (**Figure 12**). Therefore, the results can be used to estimate the concentration of seed (PrP^{Sc}) and/or makes correlate to the infectivity. **Figure 12** showed the amyloid seeding rate for an endpoint dilution series of ovine BSE-infected brain homogenate between 10^{-4} and 10^{-10}. The results from the uninfected ovine brain (10^{-4} dilution) were shown to be indistinguishable from the seed at dilutions 10^{-9} and 10^{-10} further supporting our assertion that the detection limit is 10^{-8}.

At the same time, a ten-fold dilution series (10% to 0.0001%) was used to compare the detection limits of WB and RT-QuIC. According to the RT-QuIC experiment, the lowest detectable limit of brain homogenate was at 10^{-8} dilution which is equivalent to 2×10^{-5} μg or 20pg (2μl per well) of brain material and the maximum detectable range was approximately 0.2μg (10^{-4}). Overall, the detection limits (regardless of sample volume used in the assay) determined from these assays suggest that WB is 52 times more sensitive than the TeSeE™ sheep/goat kit (ELISA) with the RT-QuIC being most sensitive means of assay requiring 2.5×10^5 times less brain sample than WB.

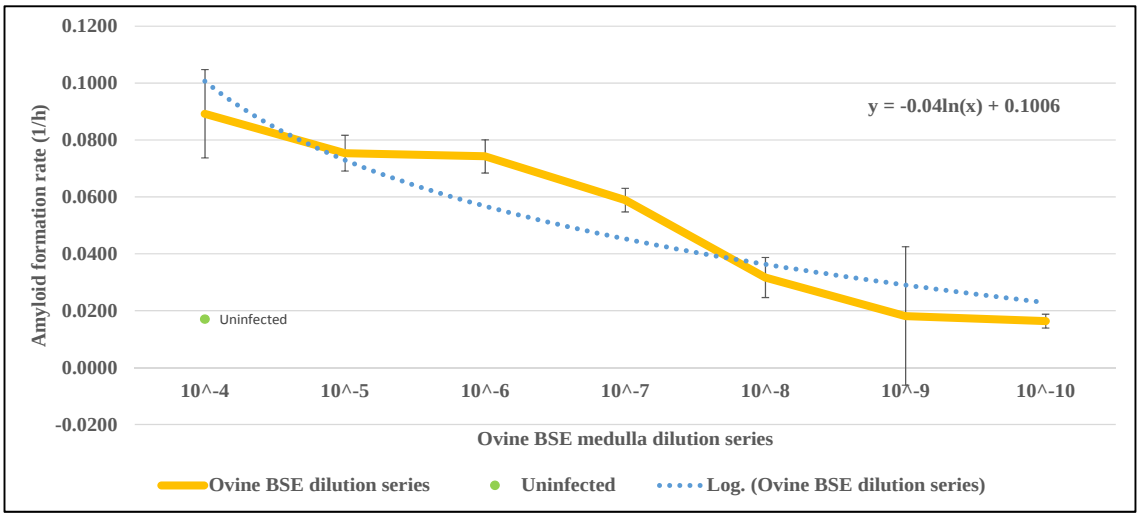

Figure 12: Amyloid formation rate (1/h) plotted against BSE-infected brain dilution series. Amyloid formation rate was determined by using the time (hours) at which average of the replicates reached the threshold (h). Inverse time (1/h) values were plotted against the BSE-infected brain dilution series on a log scale. The mean SD derived from four replicates is displayed for each dilution.

Discussion and conclusion

The TeSeE™ sheep/goat kit and IDEXX Herd-Check™ BSE EIA are the EU approved rapid kits used for PrP^{Sc} detection in small ruminants. According to the manufacturer's instructions, the TeSeE™ sheep/goat kit uses proteinase K treatment prior to immunoassay detection by two types of anti-PrP antibodies while the IDEXX Herd-Check™ BSE EIA used a ligand capture system called Seprion to detect PrP^{Sc}. The TeSeE™ sheep/goat kit was used for this study because of the similarity to WB in terms of the treatment of PrP^{Sc} with Proteinase K. Since there is no existing standard panel or calibrator available for this ELISA kit, truncated ovine $A_{136}R_{154}Q_{171}$ rPrP (rPrP) was selected as the calibrator resembling the PrP^{Sc} fragment. The relative amount of PrP^{Sc} or PrP^{Sc} level (rPrP-to-total protein equivalent) was calculated as ng/mg unit in the study. After screening for the suitable amount of rPrP for use in the quantitative ELISA (data not shown), results showed that only the 20% (w/v) concentration of lymphoid tissue homogenate was appropriate for analysis by the quantitative ELISA test – this is similar to the previously suggested concentration for screening of potentially BSE-infected samples by ELISA (*146*). Nevertheless, because of the heterogeneity of the different samples, the dilution curve for the lymphoid tissue homogenate showed minor variations compared to brain (Figures 5 and 6). The fact that the dilution curve for spleen showed that only the 20% (w/v) samples resulted in signals sufficiently greater than the lower cut-off indicated that peripheral tissues might need to be analysed at higher concentrations to enable accurate measurement of PrP^{Sc} by quantitative ELISA. The findings of a follow-up experiment (**Appendix 4**) showed that overloading of 20% (w/v) spleen homogenates into the same microplate well seemed to increase OD signals for all three genotypes by at least two fold. The increase in OD signal associated with the double loading of spleen showed that there was no obvious inhibitory effect unless otherwise stated when using the TeSeE sheep/goat kit. In addition, batches of microplate from the TeSeE sheep/goat kit tend to degrade over time following opening of the package because of the increase in humidity during storage (shelf life is specified as only 30 days). As a result, the sensitivity and specificity of the coated microplate can be reduced. Therefore, quality control measurements (intra-assay and inter-assay CVs) were obtained to demonstrate that the results obtained for data analysis were valid in precision and reproducibility.

Although there was some inconsistency in results for brain, no particular trends were identified for any genotype group, perhaps because quantitative ELISA might not be the most sensitive assay to detect if there is any difference in relative PrP^{Sc} levels among codon 141 genotypes.

Surprisingly, the results from the quantitative ELISA showed that the relative amounts of PrP^{Sc} (referred to as PrP^{Sc} level) or rPrP-to-TP (ng/mg) were not significantly different between homozygotes and heterozygote in the cases of 5% and 10% (w/v) brain samples. This finding is in line with a previous publication that used densitometric analysis of WBs of brain homogenates to compare PrP^{C} and PrP^{Sc} levels (*23*). However, unlike the brain samples, spleen and PSLN samples from animals heterozygous at codon 141 repeatedly displayed relative PrP^{Sc} levels below the cut-off value (2.4 ng/mg). The deposition of PrP^{Sc} (characteristic of TSE disease) has been described as non-uniform throughout lymphoid tissues (*82*).

In vitro amplification assays such as PMCA and RT-QuIC are growing in popularity for artificially inducing PrP^{Sc} aggregate formation within a controlled, cell-free environment (*104, 105*). The determination of substrate (rPrP) and optimal reaction settings, particularly the reaction temperature, is vital for producing uniform results for amyloid seeding amplification of ovine BSE. Besides the substrate types and reaction temperature selection, plans for improvements in the RT- QuIC assay include altering salt concentration, SDS concentration, microplate shaking speed and/or shaking-to-resting intervals (*125, 131*). An alternative approach to improving the sensitivity for PrP^{Sc} detection in blood is to freeze-thaw the blood components at least three times (to release the membrane-bound PrP^{Sc} from the blood cells) followed by treatment with sodium phosphotungstate (NaPTA) prior to RT-QuIC (*130*). The measurement of SD_{50} correlated to LD_{50} (infectivity) in various TSE-infected brain homogenates using an endpoint dilutions method has proven as a suitable quantitative method (*132, 135*).

Two types of serial dilutions (two- and ten-fold) were prepared by diluting BSE-infected brain using non-BSE sheep homogenate. The lowest detectable limit for WB was approximately 5µg of BSE brain equivalents loaded in each lane (10µl). The detectable range for WB was between 5µg to 5,000µg. This result was actually similar to the detection limit observation of 3µg of vCJD brain equivalent (*147*). Besides that, use of the ROS-BC6 anti-PrP antibody to detect PrP^{Sc} in ovine BSE samples was shown to be more sensitive than Sha 31[p] anti-PrP antibody (*148*). Moreover, ROS-BC6 and Sha31 anti-PrP antibodies detect a similar PrP protein epitope (^{148}YEDRYYR^{154}) in the cases of ovine and bovine BSE (*149*). In other words, this also means that our WB detection protocol (with NaPTA precipitation method) was more sensitive than TeSeE WB (*148*).

[p] Detects bovine BSE sample at the 30µg of equivalent brain material.

According to the endpoint dilution analysis, the lowest detectable limit of brain homogenate (ovine BSE) in the RT-QuIC was the 10^{-8} dilution, which is equivalent to 2 x 10^{-5} µg (2µl per well) brain equivalents. Different studies have used endpoint dilution analyses to demonstrate that the detectable limit for 263K was at 10^{-7} dilution (*132*), RML mouse-adapted scrapie was detectable to 10^{-8} dilution (*124*), sCJD was detectable to 10^{-9} dilution (*119*), and TSE disease in deer (CWD) was detectable in a brain sample at 10^{-8} dilution (*135*). Collectively, the endpoint dilution analysis of TSE infected samples indicated that the detectable range of PrP^{Sc} in the brain is between the 10^{-7} and 10^{-9} dilutions depending on the species in question. Our study suggested that the RT-QuIC was the most sensitive assay to detect PrP^{Sc}, compared to ELISA and WB. RT-QuIC required 2.5 x 10^5 times less brain material in the analysis than WB and was 1.3 x 10^7 times more sensitive than the TeSeE™ sheep/goat kit (ELISA).

ELISA is the most rapid technique for detecting and quantifying the relative PrP^{Sc} levels since it generally provides a result within 8 hours and has the advantages of consistency in the reagents. The drawback for ELISA was that detection of PrP^{Sc} is limited to the brain and peripheral tissues. On the other hand, the Western blot analysis method requires at least three consecutive days of "hands-on" time to detect PrP^{Sc}. Although WB is a time-consuming technique it offers consistency in the detection of misfolded PrP^{Sc} and has a wider detectable range compared to ELISA. RT-QuIC is the most sensitive assay for detection of the amyloid seeding (PrP^{Sc}) process but the downside of the technique is the requirement of at least two weeks to produce a suitable substrate (rPrP). RT-QuIC needs at least one day of experimental time for completion of the analysis though both WB (after PrP^{Sc} amplification with PMCA) and RT-QuIC can be used for detection from a wider range of samples including body fluids such as blood. Both *in vitro* conversion assays (RT-QuIC and PMCA) are proven reliable assays for detecting PrP^{Sc} from body fluids such as blood, urine and saliva samples (*73*, *114*, *129*, *135*).

Generally, a low level of PrP^{Sc} requires a longer incubation time for replication (also termed conversion) before reaching the threshold (similar to the RT-QuIC mechanism) and this will ultimately delay the progress of the disease as it begins to reach the CNS (referred to as the neuroinvasion event). The naturally occurring PrP genotypes show differences in their conversion rates (*25*). Although results from the *in vitro* conversion assays showed that the conversion rates for PrP^{C} to form PrP^{Sc} amyloid were comparable between L_{141} and F_{141} homozygotes, a closer look at the results indicates that F_{141} actually was converted with a slightly

reduced rate. This observation can be linked to the longer disease survival time compared to the wild type (L_{141}) animals (*25*). The difference in relative PrP^{Sc} levels according to codon 141 genotype was suggested as a contributor to disease susceptibility and/or incubation time in sheep infected with BSE (*23*). Numerous studies have identified that the TSE diseases incubation time is determined by the host genotype (*30, 150-152*). Natural transmission of scrapie was observed along with PrP^{Sc} deposition in sheep with the susceptible genotype (homozygote $V_{136}R_{154}Q_{171}$) only while no PrP^{Sc} deposition was found in homozygote $A_{136}R_{154}R_{171}$ or heterozygote $V_{136}R_{154}Q_{171}$/ $A_{136}R_{154}R_{171}$ animals (*30*). Oral challenge of sheep with bovine BSE illustrated the involvement of GALT, lymphoid tissues and the nervous system in BSE (PrP^{Sc}) dissemination in *PRNP* homozygote $A_{136}R_{154}Q_{171}$ animals but not in homozygote $A_{136}R_{154}R_{171}$ animals (*34*).

Collectively, the influence of a single polymorphism (codon 141) of the *PRNP* gene on the PrP^{Sc} replication rate potentially plays a key role in determining the incubation period of BSE in sheep. The PrP^{C} to PrP^{Sc} conversion process is vital in the transmission of TSE diseases, a process that mainly depends on the conversion efficiency between PrP^{Sc} strains of different species and the host PrP^{C} at the molecular level (*153*). There is evidence to suggest that bovine (cattle) BSE infection in host ovine (sheep) plays an important role in the PrP^{Sc} conversion process – this is also known as the species barrier (*154*). In our previous study, the sheep challenged with bovine BSE via the oral route was shown to have genotype-dependent disease incubation times (*23*).

A similar observation of genotypic effect on transmission has been shown in an experiment where sheep had been infected with scrapie through a stomach tube that the results showed that the *PRNP* genotypes of donor and recipient influence the disease transmission (*155*). Transmission of disease between the same genotype was successful but not among different genotypes (*155*). The results from a study of the conversion rate in ovine BSE among sheep with different *PRNP* genotypes showed that ovine BSE ($A_{136}R_{154}Q_{171}$) has a wider range of conversion rate (*156*). As predicted from the polymerisation-based model, these findings have proven that being heterozygous can interfere with the conversion process (*153*). This result indicated a heterozygote at codon 141 with a much lower amount of relative PrP^{Sc} might be a key to determining the BSE incubation time due to its effects on conversion rate.

According to the previous study (*23*), densitometric analysis of immunoblots showed that levels of both PrP^{Sc} and PrP^{C} in the brain did not differ significantly according to codon 141 genotype

(*23*). Therefore, we proposed that PrP^{Sc} replication in lymphoid tissues might have a major influence on the incubation time of BSE infection in sheep. The hypothesis was also based on our previous histopathology findings that demonstrated that heterozygote animals have less PrP^{Sc} deposition in the lymphoid tissues (spleen, PSLN, MLN, tonsil and DiPP) compared to homozygote animals (unpublished data). The ultimate aim for future work would be to investigate the relationship of relative PrP^{Sc} level in lymphoid tissues and blood components to see if this correlates with BSE infectivity by making use of the RT-QuIC seeding dose method (*135*). The infectivity titre of TSE disease is usually assessed by bioassay either by using an animal model, such as a rodent, ruminant or non-human primate, or by using a cell-based assay (*61*, *71*, *72*, *84*, *138*). Scientists have successfully employed cell-based assays as alternatives to the animal model (*157*). Thus, it is possible to assess the infectivity of ovine BSE-infected samples using cell-based assays. Due to the time frame and resource limitations, development of cell-based assays will be addressed in due course. Additionally, infectivity of prion disease does not always correlate with PrP^{Sc} level which does indicates the involvement of other factors. Therefore, comparing the RT-QuIC seeding dose (SD_{50}) with the lethal dose (LD_{50}) from bioassay is the solution used by many to determine the infectivity titre (*132*, *133*, *135*).

To conclude, truncated ovine $A_{136}R_{154}Q_{171}$ recombinant PrP was used as the calibrator (standard) in a quantitative ELISA (TeSeE™ sheep/goat kit) and as the substrate in the RT-QuIC for detection of ovine BSE. In this study, PrP^{Sc} levels were observed to be < 2.4ng/mg (cut-off) for *PRNP* heterozygote LF_{141} samples (spleen and PSLN) compared to homozygote (L_{141} and F_{141}) but no differences were seen among brain samples from the different genotypes. This evidence suggests that the different levels of PrP^{Sc} in the periphery between homozygotes and heterozygotes might affect the onset of BSE disease, especially PrP^{Sc} replication in the lymphoid tissues (GALT). Since quantitative ELISA was not a highly sensitive method for comparing relative PrP^{Sc} levels in lymphoid tissues, other assays such as quantitative WB and RT-QuIC can be an alternative method for determining the PrP^{Sc} levels in the lymphoid tissues. The detection limit in brain tissue for ELISA (TeSeE™ sheep/goat kit was 260.4µg (per 100µl), for Western blot it was 5µg (per 10µl) and for RT-QuIC it was 0.02ng (per 2µl) when making use of the rPrP substrate at the $50^{o}C$ reaction temperature.

Future plan and implication

For future work, we would aim to use quantitative WB and/or RT-QuIC to confirm our observations of the peripheral tissues and blood components. Additionally, due to the critical role of rPrP as a calibrator, the stability of the recombinant protein during storage requires further analysis. Further validation of the results requires study of PrP^{Sc} levels in other lymphoid tissues such as mesenteric lymph node and distal ileal Peyer's patches. To confirm the PrP^{Sc} profile from the RT-QuIC experiment, while avoiding a false positive reaction, further confirmation of the PrP profiles of the samples from Western blots is clearly essential. An alternative to RT-QuIC, PMCA would be a study suitable for low levels of PrP^{Sc} not only in tissues but also in blood components. To date, no suitable cell line has been found for determining the infectivity titre of ovine BSE and it would therefore be beneficial to develop an ovine BSE-sensitive cell line. Nevertheless, even without a cell-based assay, an alternative approach for investigating the infectivity titre of ovine BSE, as well as correlation with the relative PrP^{Sc} level, would be to make use of an endpoint dilution study on transgenic mice (TgARQ mice).

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Appendixes

1. Buffer and reagent composition

MicroBCA
Reagent A
The combination of sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M NaOH.

NaPTA precipitation
NaPTA solution
4% w/v NaPTA stock (Sigma, P6395) was prepared in the distilled water and titrated to pH 7.4 with NaOH before usage at a final concentration of 0.3% (w/v) containing 170mM MgCl2.

SDS-PAGE
Protein molecular markers
3µl of MagicMark™ XP Western Protein Standard (Invitrogen™, LC5602) mixed with 10µl Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (Bio-Rad, 1610375).

rPrP protein production
Lysis buffer
20µl lysozyme per ml, sodium deoxycholic acid final volume at 1mg/ml and 5µ DNase per ml.

Ni-NTA Superflow
Equilibrated in denaturing buffer pH 8.0 - 100 mM sodium phosphate, 10 mM Tris, and 6 M guanidine-HCl.

Renaturing buffer
100 mM sodium phosphate (pH 8.0) and 10 mM Tris.

Elution buffer
100 mM sodium phosphate (pH 6), 10 mM Tris, and 500 mM imidazole.

Dialysis buffer
10mM sodium phosphate pH 5.8 pre-filtered with 0.2 mm filter.

RT-QuIC reaction setting
Reaction buffer
1X PBS, 170mM NaCl, 1mM EDTA, 10µM ThT, 0.1mg / ml rPrP.

2. Intra-assay CV (%) for all ELISA experiments

	Intra-assay CV (%)	
Experiment	**Sample size (N)**	**Intra-assay CV (%)**
A	16	3.02
B	32	8.58
C	32	6.63
D	32	6.42
E	16	5.82
F	8	5.01
G	7	2.75
H	11	4.26
I	3	5.33
J	3	4.20
K	7	4.21

3. Comparison of PrP^{Sc} levels in BSE-infected brains of the different *PRNP* 141 genotypes.

Sheep ID	***PRNP* codon 141 genotype**	**rPrP-to-TP (PrP^{Sc} level, ng/mg)**			
		10% (w/v) brain		**5% (w/v) brain**	
		Experiment A	**Experiment B**	**Experiment B**	**Experiment C**
N258	LL_{141}	24.5	70.7	64.0	50.3
N261	LL_{141}	22.6	76.4	79.4	53.0
N233	LL_{141}	33.1	88.7	54.9	< 2.4
N231	LL_{141}	nd	51.7	64.3	nd
N236	LL_{141}	11.7	138.8	< 2.4	28.7
N206	FF_{141}	8.1	43.3	< 2.4	25.8
N228	FF_{141}	high	122.7	216.1	113.2
N180	FF_{141}	39.4	87.6	62.7	63.7
N218	FF_{141}	44.4	158.2	53.2	41.9
N226	FF_{141}	high	22.9	< 2.4	38.0
N220	FF_{141}	55.5	65.9	98.7	31.1
N264	LF_{141}	25.1	31.8	127.1	< 2.4
N248	LF_{141}	high	68.6	60.4	61.1
N245	LF_{141}	36.5	92.8	135.2	< 2.4
N204	LF_{141}	13.2	110.6	52.6	< 2.4
N223	LF_{141}	high	70.8	< 2.4	23.1

"nd" denotes that sample was not done in the experiment, whilst "high" indicates that results were above the linear range. The results indicated as high or <2.4 were excluded from data analysis.

4. **Comparison between single and double loading of the BSE-infected spleen.**

Percentage (w/v)	20%	20% + 20%
Spleen 1	0.049	0.152
Spleen 2	0.157	0.359
Spleen 3	0.009	0.022